Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 104 AUGUST-SEPTEMBER, 1960 No. 4

SECTION MEETINGS

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IOWA	
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Hypocholesteremic Effect of β-Diethylaminoethyl Diphenylpropylacetate Hydrochloride (SKF No. 525-A) in the Dog. (25896)

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In view of general acceptance of relationship between hypercholesteremia and incidence of atherosclerosis, agents capable of reducing plasma cholesterol levels are of considerable current interest. In this laboratory β - diethylaminoethyl diphenylpropylacetate hydrochloride (SKF No. 525-A) was a potent hypocholesteremic agent. This report describes results of *in vivo* tests with this compound.

Methods. Adult mongrel dogs with naturally-high plasma total cholesterol values (greater than 150 mg%) were used. Dogs of both sexes were distributed evenly throughout the test. A commercial dog food and enriched whole milk diet sufficient to maintain body weight(1) was fed once a day (morning), and water supplied *ad lib.;* food not consumed within 3 hours was removed. Animals were housed individually in raised, wide-mesh screen cages in room at constant temperature and humidity. Medication was given orally in gelatin capsules, twice a day on weekdays (following 11:00 a.m. feeding and at 4:00 p.m.), and once a day on Saturday and Sunday after 11:00 a.m. feeding, so that on weekends animals received half usual daily dose of test material. Control dogs were administered an equal number of empty capsules when compound was given to test dogs. Blood samples were obtained from brachiocephalic vein of test dogs in fasting condition (before morning feeding). In Exp. I, 5 dogs received SKF No. 525-A in 20 mg/kg body weight/day for 2½ weeks, while 5 comparable dogs were given placebo treatment. In Exp. II, 3 dogs received the compound at 10 mg/kg/day for 19½ weeks, and 5 dogs again served as controls. Plasma total cholesterol was studied at intervals; plasma total lipids, phospholipids,

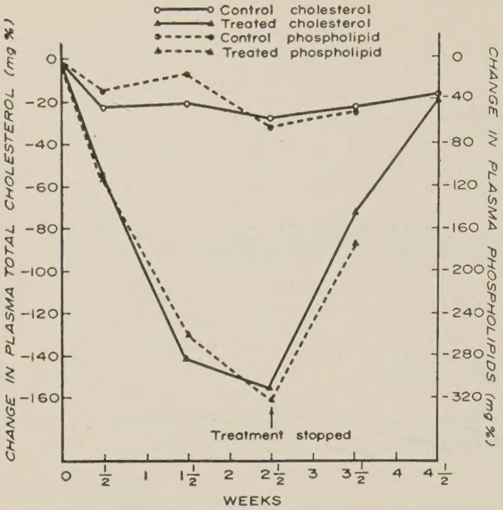

FIG. 1. Changes in plasma total cholesterol and plasma phospholipid in dogs treated with 20 mg SKF No. 525-A/kg/day (Exp. I). At start of experiment (0 time) absolute plasma base values in mg % were: Control cholesterol, 238; SKF No. 525-A treated cholesterol, 186; control phospholipid, 466; SKF No. 525-A treated phospholipid, 403.

esterified cholesterol and free cholesterol were studied at or near termination of experiment. A thyroid function test (protein-bound iodine) and liver function tests (serum alkaline phosphatase, bromsulfalein clearance and serum bilirubin) were determined after 17 weeks of drug administration. Other tests included glucose tolerance, complete blood study, urinary sodium and potassium, and urinary creatine/creatinine. Dogs were then autopsied for gross examination of internal organs, histological studies, and lipid analyses of livers and aortas. Cholesterol analyses were done by modification of method of Anderson and Keys(2). Other determinations were done by standard procedures(3-6).

Results. In Exp. I, 20 mg of the compound/kg/day administered orally to dogs resulted in an 84% reduction of plasma total cholesterol in 2½ weeks. Upon withdrawal of the compound these values returned to pretreatment levels (Fig. 1). Plasma phospholipids followed a pattern similar to that of the plasma total cholesterol (Fig. 1). Results of Exp. II (Table I) show similar reduction of plasma total cholesterol, plasma total lipids, and plasma phospholipids in dogs treated with a lower level of test material (10 mg/kg/day). Although studies during fifth month of treatment (Exp. II) showed that serum bilirubin, bromsulfalein retention and per cent esterified cholesterol were not significantly altered in treated dogs, serum alkaline phosphatases were significantly higher than for untreated dogs (Table I), indicating altered

TABLE I. Dog Exp. II. Effect of Oral Treatment of Dogs with 10 mg SKF No. 525-A/kg/Day for 19½ Weeks on Plasma Lipids, Liver and Thyroid Functions, and Body Weights.

Measurement	Control dogs (5)	Treated dogs (3)	"P"*
Plasma lipids, mg %			
Total cholesterol			
Initial	231 ± 76	216 ± 53	.8
After 3½ weeks' treatment	215 ± 56	88 ± 47	.02
" 19½ " "	245 ± 66	92 ± 16	.005
Free cholesterol, 17½ wk	66 ± 20	17 ± 8	.02
Esterified cholesterol, 17½ wk	200 ± 63	73 ± 10	.05
% esterified (of total), 17½ wk	76 ± 1	81 ± 5	.5
Total lipids, 19½ wk	1097 ± 89	770 ± 73	.001
Phospholipids, 19½ wk	406 ± 63	183 ± 28	.005
Cholesterol/phospholipid ratio, 19½ wk	.55 ± .05	.50 ± .04	.5
Liver function tests (17 wk)			
Serum alkaline phosphatase, Bodansky units	1.3 ± .9	7.9 ± 2.1	.001
Bromsulfalein, % retention	10.9 ± 6.0	12.9 ± 7.9	.5
Serum bilirubin, mg %	.10 ± .08	.15 ± .05	.5
Thyroid function test (16½ wk)			
Protein-bound iodine, μg %	2.1 ± .6	1.2 ± .1	.05
Body wt, kg			
Initial	12.5	11.0	
Final	12.3	11.1	

* "P" value determined by Student 't' test comparing values obtained with treated dogs to those with control dogs at times indicated.

TABLE II. Dog Exp. II. Lipid Analyses of Livers and Aortas of Dogs Treated with 10 mg SKF No. 525-A/kg/Day for 19½ Weeks.

		Livers			Aortas,
Dog No.	Treatment	% dry wt	% fat (dry wt)	mg chol/g dry wt	mg chol/g dry wt
165	None	32.0	4.7	7.8	5.8
208	"	31.0	4.1	7.2	5.8
	Avg	31.5	4.4	7.5	5.8
171	SKF No. 525-A	33.2	22.5	9.0	4.0
213	*Idem*	32.4	19.5	9.3	4.7
	Avg	32.8	21.0	9.2	4.4
54	SKF No. 525-A 19½ wk, removed from treatment for following 3 wk	30.4	5.4	7.3	4.9

liver function(7). Protein-bound iodine levels of test animals were significantly lower than were those of control dogs. The following tests gave normal values in treated dogs: glucose tolerance (19½ wks), urinary creatine and creatinine (6 wks), urinary sodium and potassium (6 wks), blood count and white cell differential (16½ wks), blood volumes (18 wks), and albumin/globulin ratios (15 wks). There were no apparent changes in appetite nor did body weights change during experimental periods.

Fatty infiltration of livers of treated dogs brought to autopsy was observed grossly and was confirmed by chemical analyses (Table II). Liver cholesterol of treated animals was 23% higher than that of controls, whereas aortic cholesterol was 24% lower. By histopathologic examination no abnormalities were observed in organs (including the thyroid) other than the liver in treated dogs.

One dog, which received SKF No. 525-A for 19½ weeks of Exp. II, was removed from treatment for 3 weeks before autopsy (Dog No. 54). In this dog plasma alkaline phosphatase level returned to normal and plasma total cholesterol increased from 35 mg% to 194 mg% (pretreatment value, 196 mg%); the liver appeared on autopsy both chemically (total lipids) and histologically normal. Although fat and cholesterol contents of this dog's liver had returned to levels similar to those of control dogs, aortic cholesterol was still depressed below control level.

Discussion. SKF No. 525-A has previously been known as potentiator of various drugs including hypnotics, analgesics, spinal cord depressants and CNS stimulants(8). The mechanism of action of SKF No. 525-A as a potentiator is related to its activity as inhibitor of side-chain oxidation, hydroxylation, ether cleavage, dealkylation, deamination(8), acetylation and cholinesterase activity(9). *In vitro* studies on rat liver homogenates, performed by Dr. W. L. Holmes, showed that SKF No. 525-A also inhibits synthesis of cholesterol from mevalonate. SKF No. 525-A, therefore, appears similar to other recently-announced hypocholesteremic agents which inhibit cholesterol synthesis from mevalonate (10,11).

Rapidity of action of SKF No. 525-A is exhibited in Exp. I where 30% reduction in plasma total cholesterol levels was produced in less than a week of treatment. The second experiment reveals that the hypocholesteremic effect is sustainable over a long period without overt signs of toxicity.

It has recently been reported that in rats long-term administration of SKF No. 525-A at high levels (25-50 mg/kg/day for 2 months) resulted in fatty infiltration of the liver(12). Doses of 10 mg/kg/day over the 2-month test caused no liver changes in rats (perhaps a longer dosage administration of lower level of the compound would have resulted also in liver changes).

Under our conditions with dogs, the alkaline phosphatase levels were more sensitive to liver changes than were bromsulfalein clearance times or serum bilirubin levels. The change in alkaline phosphatase was the only indication of hepatic toxicity, which suggests that liver damage with a drug of this type might be overlooked if reliance were placed only on such standard liver-function tests as per cent esterified cholesterol, bilirubin levels or BSP clearance.

Tests on the dog when treatment was stopped after 5 months, indicate ready reversibility of fatty infiltration of the liver. It is therefore possible that intermittent use of the drug might lower serum cholesterol without causing excessive fatty livers. The prompt return of plasma cholesterol to pretreatment levels, observed in Exp. I and II upon cessation of treatment, indicates lack of permanent alteration of cholesterol-synthesizing mechanisms.

Additional work in this laboratory with species other than the dog showed that SKF No. 525-A produces hypocholesteremic effects in rats(13), mice and monkeys. In both rats and mice treated with SKF No. 525-A, we observed fatty infiltration of liver; monkeys were not autopsied for hepatic examination.

Summary. β-Diethylaminoethyl diphenylpropylacetate hydrochloride (SKF No. 525-A) reduced significantly plasma total cholesterol and aortic cholesterol in dogs. Chronic daily administration of the SKF No. 525-A to dogs for 5 months resulted in marked fatty infiltration of the liver, which was rapidly reversible upon withdrawal of the compound.

The authors wish to thank Dr. C. A. Leonard for drug studies in monkeys and Dr. L. Z. Saunders for histopathological studies.

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Received November 18, 1959. P.S.E.B.M., 1960, v104.

Further Evidence of Immunologic Dissimilarity of Distemper (CD) and Measles (M) Viruses.* (25897)

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Although a possible relationship between canine distemper (CDV) and measles (MV) viruses has been proposed by various investigators(1-5), others find experimental evidence against such a theory(6,7). Inasmuch as modified canine distemper virus has been suggested for use in immunizing children against measles(8), further investigation of the relationship of these 2 viruses is required. Additional experimental evidence of their dissimilarity is presented here.

Materials and methods. Virus strains used: Edmonston strain† of MV adapted to chick embryo tissue culture(9); HeLa-grown MV with titers of $10^{4.5-5.5}$ TCD_{50} for inoculation of monkeys and ferrets and for neutralization tests; Lederle chick-embryo adapted CDV (10) for chick-embryo tissue culture and neutralization tests and for vaccination of ferrets. Virus stock was prepared as described earlier (6). Ferrets were challenged with Lederle virulent CDV(11). *Tissue culture.* Growth medium consisted of Eagle's solution(12) containing lactalbumin hydrolysate, to which was added: 2% calf serum for monkey kidney cultures, 10% horse serum for chick embryo and 30% calf serum for HeLa cells, all prepared by the method of Dulbecco and Vogt (13). Renewal fluids were medium 199(14) plus 2% hydrolyzed gelatin and 1% liver digest for monkey kidney cultures; medium 199 alone for chick embryo; and Eagle's solution plus 20% horse serum for HeLa cultures. *Human sera.* Thirteen paired acute and convalescent measles sera and 3 convalescent sera collected during a 1947 epidemic in Cleveland[‡] were stored in a dry ice cabinet until tested. *Measles immune monkey serum.* Serial bleedings from cynomolgus monkeys immunized with MV[§] were tested for MV and CD neutralizing antibodies. *Neutralization tests.* CD antibodies were titrated in chick embryos(6). MV neutralization tests were carried out as follows: 2-fold dilutions of inactivated serum (30 minutes at 56°C) and approximately 100 TCD$_{50}$ of virus were mixed and incubated one hour at room temperature. Each of 2 HeLa culture tubes was inoculated with 0.1 ml of each serum-virus mixture, incubated for 14 days at 37°C and examined daily for multinucleated cells characteristic of MV. Renewal fluid was changed every 4th day. Paired serum specimens were always tested at the same time, and normal and immune serum controls as well as a virus titration, were included in each test. Serum neutralizing titers (SN$_{50}$) were calculated by Reed and Muench formula(15). *Control immune sera* were the same as those used in a previous study(6).

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* Presented in part at SAB meeting in Philadelphia, May 1, 1960.

† Courtesy of Dr. J. F. Enders, Children's Hosp., Boston.

Results. Immunization of Ferrets with MV. Blood samples from 4 groups of 3 ferrets were pooled by group. Two groups were then inoculated with a HeLa-grown MV suspension according to following schedule:

Day	Route	Dose
0	IM in 2 sites	2 ml MV-adjuvant mixture[‖]
	IP	1 ” [¶]
7	IP	1 ”
14	IM in 2 sites	2 ” -adjuvant mixture
	IP	1 ”
21	IP	1 ” (Test bleeding done on this day.)

[‖] MV-adjuvant mixture: 5 parts virus suspension, 4 parts Bayol F, and 1 part Arlacel A.

[¶] MV: virus suspension—$10^{4.5}$ to $10^{5.5}$ TCD$_{50}$/ml.

The other 2 groups were inoculated according to the same schedule with a normal HeLa cell preparation. Sera from 2 ferrets were pooled and the ferrets then vaccinated with modified CDV. On day 21, all ferrets were re-bled and their sera pooled by group. CDV and MV neutralizing antibody titers on all pools had been determined by day 31, and third blood specimens were obtained on that day before the animals were challenged with virulent CDV. One MV, one HeLa control and the CD vaccine groups of ferrets received 10,000 ferret LD$_{50}$, and the other MV and HeLa groups received 1,000 LD$_{50}$ of CDV. All serum pools were again tested for CD and MV neutralizing antibodies (Table I). With use of adjuvant, demonstrable MV antibodies were elicited in MV inoculated ferrets, yet no CD antibodies were formed and all animals succumbed to CDV challenge. CD vaccination induced CD antibodies and immunity to challenge, but did not induce MV antibodies. *MV and CDV SN$_{50}$ titers from 16 measles patients.* Time of bleeding and virus titers are given in Table II. Variable amounts of available serum account for the different starting dilutions. Appearance of MV and CD antibodies in the same individual are clearly not correlated. The highest CDV levels (between 1:10 and 1:30) were found in sera from 2 patients, of whom one had no history of clinical measles, and from MV-immune monkeys. No significance is attributed to CD titers of less than 1:20, as dogs with even slightly higher titers have been found fully susceptible to distemper challenge(16). Ac-

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[‡] Courtesy of Dr. A. B. Sabin, Children's Hosp., Cincinnati.

[§] Data to be published.

TABLE I. Immunization of Ferrets with MV and Subsequent CDV Challenge.

Ferret group No.	Inoc.	Neutralizing antibodies MV/CD Pre-vac.	21 days	31 days	Mortality ratio after CD challenge
1	HeLa control	<4/<5*	<4/<5	<4/<5	3/3†
2		”	”	”	” ‡
3	HeLa MV	”	<60/<5	20/<5	” †
4		”	60/<5	10/<5	” ‡
5	CD vaccine	”	<4/1800	<4/1300	0/2

* Reciprocal of dilution. † Challenge dose 10,000 LD_{50} CDV. ‡ Challenge dose 1,000 LD_{50} CDV.

tively immune dogs often possess titers of 1:1000 or more, as illustrated by the control CD-immune dog serum, which was devoid of measles antibodies. Judging from convalescent phase titers, all but 2 patients had had clinical measles, perhaps even the 3 from whom no acute phase sera were available. Yet distemper antibodies are absent or insignificant in all. *Effect of homologous and heterologous antisera on multiplication of MV and CDV.* MV was grown in 3 groups of HeLa culture tubes. In one group the medium contained 5% normal horse serum; in the second group, 5% control MV-immune monkey serum; in the third, 5% control CD immune dog serum. After 14 days incubation at 37°C, pooled fluids from each group were titrated in HeLa cultures. Groups of chick-embryo culture tubes were renewed with medium 199, alone or plus one of the following: 2.5% normal dog serum, 2.5% control CD-immune dog serum, or 2.5% control MV-immune monkey serum. Tubes were inoculated with tissue culture adapted CDV(17) and incubated for 4 days at 37°C. Tissues and fluids from each group were harvested, ground together and titrated on the CAM of 7-day-old chick embryos (Table III). MV growth was significantly inhibited (3 logs) by the homologous, but not at all by the heterologous sera. MV-antiserum in the medium did not interfere with CDV growth, but CD-immune serum did. *MV and CD antibody titers of measles-immune monkeys.* One of 6 susceptible monkeys developed MV antibodies one week after vaccination, but died of pneumonia on the 13th day. The others developed MV titers by the second week post-vaccination and maintained them at high levels for 6 weeks of observation. Except for animals Nos. 4 and 5, which lacked significant CD titers, CD antibodies were absent despite the

TABLE II. Distemper and Measles Antibody Titers of 16 Measles Patients.

Clinical signs at first bleeding	Serum neutralization titers* Acute/Convalescent MV†	CD‡
Koplik spots	<8/44	<5/ 10
	<8/64	<25/ 5
	<8/90	<5/ 5
	<4/20	<5/<5
	<8/64	<25/<5
	<8/90	<5/<5
	<4/128	<25/<5
	6/80	”
Rash—2nd day	10/64	<5/ 5
3rd ”	8/8	”
Idem	10/64	”
”	8/32	”
Not obtained	—/40	—/<25
Idem	—/128	—/<5
”	—/128	”
No clinical measles	64/90	10/ 30
Control measles immune monkey serum	140	20
Control distemper immune dog serum	<4	4,000

* Reciprocal of dilution.
† Tested against 100 TCD_{50} of virus.
‡ ” ” 100-500 CED_{50} of virus.

TABLE III. Multiplication of MV and CD Viruses in Presence of Homologous and Heterologous Antisera.

Type serum added to medium	MV titers in HeLa cells	CD titers in chick embryos (EID_{50}/ml)
None		4.2
Normal horse	5.5*	
” dog		4.2
MV-immune monkey	2.5	4.3
CD- ” dog	5.5	Neg. und.

* Log to power of 10.

TABLE IV. Measles and Distemper Antibody Titers of Cynomolgus Monkeys Immunized with Measles Virus.

Time of bleeding (wk)	Monkey No. and serum neutralizing antibodies 1 CD	1 MV	2 CD	2 MV	3* CD	3* MV	4 CD	4 MV	5 CD	5 MV	6 CD	6 MV
0	<5†	<4	<5	<4	<5	<4	<5	<4	<5	<4	<5	<4
1	"	"	"	"	"	44	"	"	"	"	"	"
2	"	40	"	20			"	30	"	30	"	44
3	"	90	"	180			"	360	10	260	"	180
4	"	"	"	260			"	"	"	510	"	"
5	"	60	"	"			"	510	<5	260	"	90
6	"	90	"	180			10	"	"	180	"	180

* Died of pneumonia on 13th day. † Reciprocal of dilution.

presence of elevated levels of MV antibodies (Table IV).

Discussion. Tests with the strains used in this study indicate little antigenic similarity between MV and CDV, and any relationship between them seems remote. Warren *et al.* (5) have suggested that strain and passage histories of CDV might bear on its degree of similarity to MV. Since only one serologic type of CDV has been recognized, such variations in the antigenic mosaic of the virus are difficult to assume. Our tests on sera from patients convalescing from measles showed no evidence of simultaneous development of CD and MV antibodies. Possibly CD antibodies develop later. Absorption experiments with human sera containing antibodies to both viruses are in progress and results should furnish further clues to their antigenic relationship.

Summary. Additional experimental evidence of dissimilarity of canine distemper (CDV) and measles (MV) viruses are presented: 1. Ferrets inoculated with repeated doses of MV-adjuvant mixture and which developed M antibodies proved as susceptible as control animals to challenge with CDV. 2. Paired convalescent sera from measles patients showed significant measles antibody rises, while CD antibodies remained essentially absent. 3. Growth of MV in HeLa cells was not inhibited by presence of CD antiserum or normal serum, while MV antiserum apparently reduced virus multiplication. Presence of measles antiserum did not interfere with propagation of CDV. 4. By 14th day after MV infection, serologically negative monkeys developed high antibody titers for MV but none for CDV. It remains to be determined whether results obtained are attributable to particular virus strains used, or apply also to other isolates of MV and CDV.

Authors thank Marie Murrin and Hubert Klaver for technical assistance and E. Manoogian for help in preparation of manuscript.

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Received April 1, 1960. P.S.E.B.M., 1960, v104.

Effect of Lactogenic Hormone, Growth Hormone and Thyroxine in the Lactating Albino Rat.* (25898)

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It has been shown that oxytocin§ (OXT), growth hormone|| (GH) and thyroxine (T4) improve significantly milk yields of lactating rats(1,2). Lactogenic hormone|| (LGH) requirements have been investigated in the intact lactating rat. These data indicated that normal milk secretion required not only LGH, but also other pituitary hormones(3). In continuing these studies, the effect of lactogen alone and combinations of lactogen, growth hormone and thyroxine upon litter weight gain and milk yield on day 14 of lactation are reported.

Materials and methods. 121 Sprague-Dawley-Rolfsmeyer lactating rats were housed, fed, and killed under identical standardized conditions as described previously(1,2). Nursing was standardized in all rats with 1 USP unit OXT given subcutaneously immediately before young were allowed to suckle. One mg LGH/day, 1 mg GH plus 1 mg LGH/day and 1 mg LGH plus 1 mg GH plus 3 μg T4/100 g body weight (BW)/day were injected from day 7 through day 14 of experiment. A 0.9% alkaline NaCl solution served as vehicle at rate of 0.2 mg/1 mg GH, LGH, or 1 USP unit OXT, and 0.1 mg/3 μg T4. Control animals were treated in same way but were injected with 1 USP unit OXT on day 14.

Results. Lactogen, 1 mg/day from day 7-14 of lactation, had no beneficial effect on prenursing litter weight and milk yield on day 14. Combination of same amount of LGH with 1 mg GH/day increased significantly prenursing litter weight and total milk yield as compared to controls and LGH treated group (Table I).

Addition of 3 μg T4/100 g/day to LGH plus GH resulted in a more highly significant increase in milk yield on day 14 in comparison to oxytocin alone than did treatment with LGH plus GH (from $p < .005$ to $p < .001$).

Litter weight on day 6 was significantly higher in group which received triple hormone treatment, as compared to controls and LGH, but not of GH treated rats. A significant increase in total litter weight on day 14 as compared to LGH treated rats, but not compared to control rats, was observed. Per cent litter weight gain on day 14 over day 6, more sensitive as a parameter, was significantly increased only in LGH plus GH treated rats.

Discussion. It was observed that OXT injected at nursing time increased milk removal 50%(1,2). Subsequently, it was shown that thyroxine at level of 3 μg/100 g BW/day from day 7 to 14 increased milk yield 37.7% in normal rats and 63.1% in conjunction with OXT. GH alone, at level of 1 mg/day from day 7 to 14 increased milk yield 41.1% over controls and with oxytocin an increase of 60.5% was observed. When GH, T4 and OXT were administered, milk yield was increased 74% over control(2).

In present experiment it was observed that injection of LGH from day 7 to 14 and OXT to aid in milk removal did not increase milk yield above that of OXT alone. However, when LGH and GH were administered together during increasing segment of lactation curve there was a significant increase from 60% with GH and OXT to 68% in milk yield with the 3 hormones. Further, with the combination of GH, T4 and OXT, milk yield was increased from 74 to 84% by addition of LGH.

These data are believed to indicate that

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* Contribution from Mo. Agr. Exp. Sta., Journal Series No. 2122.

Approved by the Director. This investigation supported in part by research grants from U.S.P.H.S.

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§ Oxytocin supplied by Armour Lab., Chicago.

|| Growth hormone, bovine and lactogenic hormone, ovine kindly supplied by Nat. Inst. Health.

TABLE I. Synergistic Influence of Lactogen, Growth Hormone, Thyroxine and Oxytocin on Milk Yield and Litter Weight Gain to 14th Day of Lactation.

Treatment (hormone, day 7-14; oxytocin, day 14)	No. of rats	Litters' avg body wt: Day 6, g	Day 14 prenursing, g	Gain g	Gain %	Avg wt of milk: Total, g	Total as % of postnursing LW	% increase over untreated controls*
OXT: 1 USP unit	38	70 ± 3.0^{1}	153	83 ± 2.9^{5}	120 ± 5.6^{9}	9.9 ± .3^{13}	6.0 ± .1^{17}	58
Idem + LGH: 1 mg/day	30	71 ± 1.7^{2}	148	77 ± 3.2^{6}	109 ± 5.2^{10}	9.4 ± .5^{14}	6.0 ± .3^{18}	58
" + " + GH: 1 mg/day	33	75 ± 2.2^{3}	168	93 ± 2.3^{7}	128 ± 4.5^{11}	11.6 ± .4^{15}	6.4 ± .2^{19}	68
" + " + " + T4: 3 μg/100 g BW/day	20	80 ± 2.0^{4}	172	92 ± 2.9^{8}	116 ± 4.7^{12}	12.8 ± .5^{16}	7.0 ± .2^{20}	84

* Data from Grosvenor & Turner(1)

Student's "t" probability:

Comparison	Probability
6-7 13-16 14-15, 16	$<.001$
2-4 13-15 17-20	$.001 < p < .005$
5-7 10-11	$.005 < p < .01$
6-8 18-20	$.01 < p < .025$
1-4	$.025 < p < .050$
5-8 11-12 15-16 17-19 19-20	$.05 < p < .1$
3-4 5-6 9-10 18-19	$.1 < p < .2$
1-3 2-3 9-11 10-12 13-14	$.2 < p < .4$

BW = Body wt
LGH = Lactogenic hormone
GH = Growth "
OXT = Oxytocin
T4 = Thyroxine

when GH and T4 stimulate increased milk yield separately and together, endogenous secretion of LGH became inadequate and additional exogenous LGH made possible the more intense lactation observed.

Summary. Effects of lactogenic hormone (LGH) alone and in combination with growth hormone (GH) and L-thyroxine (T4) upon lactation in the rat have been studied. All rats were nursed on day 14 of lactation with aid of 1 USP unit of oxytocin (OXT) under standardized conditions. One mg LGH alone did not show galactopoietic effects when injected daily from day 7-14. Under combined action of 1 mg LGH and 1 mg GH/day from 7-14, litter weight gain and amount of milk on day 14 have been significantly increased. Addition of 3.0 μg T4/100 g body weight/day from day 7-14 to LGH plus GH resulted in a more highly significant increase in milk yield in comparison to oxytocin alone than did treatment with LGH plus GH. The increase in mean milk yield of 84% over untreated controls by 4 hormones is the highest so far attained in laboratory.

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Received April 8, 1960. P.S.E.B.M., 1960, v104.

Histamine in Intestinal Lymph of White Rat During Anaphylactic Shock.* (25899)

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Histamine has been shown by Mota(1) to appear in blood of sensitized white rats within a few minutes after intravenous administration of the specific antigen, horse serum. Strickland(2) later used egg albumin and *Bordetella pertussis* vaccine as sensitizing agents and also demonstrated an increase of histamine in blood of shocked rats. Sanyal and West(3) showed that severe shock can be produced consistently in the rat with *B. pertussis* vaccine together with egg albumin or horse serum. Subsequently, Sanyal and West(4) interpreted their findings as indicating that neither histamine nor 5-hydroxytryptamine participate in symptoms of anaphylactic shock in the rat. This conclusion was reached because shock ensued in animals even after skin and intestines presumably had been depleted of histamine and 5-hydroxytryptamine by preliminary treatment with polymyxin B sulfate, reserpine and total body irradiation. Neither mepyramine maleate nor BOL 148 seemed to reduce intensity of shock. Mota(1), however, had noted previously that antihistaminic drugs protected rats against anaphylactic shock. Parratt and West(5) suggested that release of histamine owing to polymyxin B sulfate is chiefly by injury to or destruction of mast cells in the peritoneal cavity and subcutaneous connective tissue. Histamine also is released from lungs. In their opinion polymyxin B sulfate releases maximal amounts of histamine and only small amounts of 5-hydroxytryptamine. Irradiation of the entire body destroys mast cells(6, 7). It is thought that irradiation depletes chiefly stomach and jejunum of histamine(4). However, Van Den Brenk(8) suggested that mast cells are resistant to radiation. Intraperitoneal injection of distilled water also destroys mast cells in the peritoneal cavity (9,10). Feldberg and Kellaway(11) and also Dale(12) pointed out that it is the quantity of histamine liberated at site of action which is important in production of pharmacologic activity. The small intestine seems to be the "shock organ" of the rat. This study was undertaken to determine whether histamine is released into the lymph that drains from gastrointestinal tract of rats during anaphylactic shock. Some preliminary studies are included regarding effect of polymyxin B sulfate, roentgen rays, and distilled water on release of histamine.

Method. White male rats of Sprague-Dawley strain weighing 150 to 350 g were used. Sensitization was carried out by simultaneous intraperitoneal administration of 25 mg of bovine gamma globulin (BGG) and 0.5 ml of *B. pertussis* vaccine. Twelve to 24 days later 10 to 12.5 mg of bovine gamma globulin was administered intravenously to induce shock. Shortly before inducing shock the mesenteric lymphatic vessel was cannulated with plastic catheter while animals were lightly anesthetized with ether, and a small catheter placed in tail vein. Lymph for use as a control was collected during immediate recovery period. The animal was then given an intravenous injection of 3 mg of pentobarbital sodium (nembutal)/100 g body weight. No. 60 plastic catheter was tied into the right external jugular vein of animals in which blood studies were done. A control blood specimen was secured from each animal and the shocking dose was then administered intravenously.

Lymph was collected for control period, usually 30 to 60 minutes, and for periods ending 6, 12, and 30 minutes after shock. Each specimen of lymph was heparinized and kept

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* This investigation supported in part by grant to Dr. C. F. Code from Louis W. and Maud Hill Family Fn.

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at 0° to 5°C until extraction, by the method of Code(13), or direct assay was carried out. Four rats were not given sodium pentobarbital during collection of lymph or during periods of shock. Histamine content of lymph of 12 rats was studied by extraction and assay. To secure specimens of at least 0.4 ml, the lymph from 2 rats was pooled. Histamine content of both lymph and blood plasma was studied in 11 rats by direct assay. Blood was collected during control period and at 6, 12, and 30 minutes after shock. It was drawn into a heparinized syringe and kept at 0° to 5°C until centrifuged at the same temperature. Plasma was separated and kept at 0° to 5°C until assay. Histamine content of lymph and blood was determined by assay using a segment of terminal ileum of the guinea pig suspended in 3 to 4 ml of atropinized modified Tyrode's solution(13). In specimens not extracted but directly assayed, each contraction considered due to histamine was blocked by mepyramine maleate. All results are expressed in terms of histamine base.

Six rats were studied after preparation with polymyxin B sulfate by the method of Parratt and West(5). The 3-day period of administration of the drug began on 10th day after sensitization. Animals were shocked on 13th day. Three rats were studied after irradiation of whole body 6 days after sensitizing dose by modification of the method of Eisen, Ellis and Wilson(6). The following factors were used in irradiation: 130 kv, 8 ma, 4½ mm aluminum filter, and distance 63 cm; 600 r was given to the back in 30 minutes and 400 r to central surface in 20 minutes. This gave a dose of 900 r to the center of body. Because only one of 12 rats so treated survived, the dose was reduced to a total of 700 r. Four rats were given this dose; 2 survived and were studied 13 days after sensitizing dose. Four rats were given 20 ml of triple distilled water intraperitoneally 6 days after sensitization with bovine gamma globulin and *B. pertussis* vaccine. One week later a study was made of lymph during shock. Because substances, other than histamine, which cause contraction of gut, were present in lymph and plasma, direct assay was only partially satisfactory and the results must be considered largely qualitative. The results of assays after extraction are more quantitative but not as exact as desired because of technical difficulties in making extracts with small amounts of lymph collected. Clinical shock was graded by dyspnea it produced and, in unanesthetized animals, by degree of restlessness as well. Gross appearance of the small intestine was used as chief pathologic criterion; the least degree of shock was indicated by slight congestion and the greatest degree by intense congestion.

Results. Bovine gamma globulin was a satisfactory antigen for sensitizing rats when given simultaneously intraperitoneally with *B. pertussis* vaccine. Animals displayed the most intense shock when shock was induced on 13th day after sensitization. The reaction of animals shocked after 21 to 24 days was much less intense.

Histamine was found in intestinal lymph in increased amounts after anaphylactic shock (Table I). This was demonstrated not only by direct assay but also by assays carried out after extraction for histamine. Histamine appeared promptly after shock, was greatly diminished after 12 minutes, and had returned to control values in 30 minutes. In general, the more intense the degree of shock, the greater the quantity of histamine found in intestinal lymph (Table I). During initial studies when direct assay was employed, animals which had little or no clinical or pathologic evidence of shock had little or no increase of histamine in plasma or intestinal lymph.

Rate of flow of lymph was carefully checked and increased 8 to 25 times during first 6 minutes after administration of shocking dose as compared to control period. The flow remained elevated or decreased slightly during second 6-minute period and returned to control rate in 30 minutes.

After shock, the quantity of histamine in lymph of animals pretreated with polymyxin B sulfate was definitely decreased (Table II). Degree of clinical shock in those animals seemed to have been modified. Flow of lymph, however, was nearly the same as in untreated animals.

TABLE I. Histamine Content of Intestinal Lymph of Rats after Anaphylactic Shock.*

Degree of shock†	Specimen, min.	Histamine,‡ μg/ml (expressed as base)
Poor	Control	.02
	6	.60
	12	.38
	30	.03
Fair	Control	.01
	6	.64
	12	.01
	30	.00
Excellent	Control	.02
	6	1.08
	12	.06
	30	.02
"	Control	.00
	6	1.12
	12	§
	30	§
"	Control	.00
	6	1.50
	12	.17
	30	.00
2 Fair 1 Good	Control	.02
	6	.40
	12	.02
	30	.02

* Each histamine study represents lymph pooled from 2 animals.

† *Excellent,* considerable intestinal congestion, labored respiration; *good,* moderate intestinal congestion, labored respiration; *fair,* fair intestinal congestion; *poor,* little intestinal congestion.

‡ Extraction procedure used in all determinations.

§ Specimen inadequate for assay.

Pretreatment with polymyxin B sulfate alone caused death in 18 of the 32 rats so treated. All surviving rats lost 15 to 30 g.

Animals pretreated with roentgen rays became ill. Only one of 12 rats survived the dose of 900 r. Two of 4 rats given 700 r survived. No histamine was found in lymph of any of the 3 animals (Table II). They appeared too ill before shock to allow assessment of degree of clinical shock. The entire intestinal tract and peritoneum were hemorrhagic in each animal. Flow of lymph was reduced in 2 animals after shock but increased 10-fold in one rat.

Intraperitoneal injection of distilled water caused shivering in each of 6 animals so treated. In 4 animals shocked, the reaction was moderate but not extreme. Histamine content of lymph in one pair of animals may have been reduced somewhat below that noted in shocked animals without pretreatment (Table II). The increased flow of lymph during shock was the same as in untreated animals.

Comment. Sanyal and West(3) observed that shock was severest in rats sensitized with the aid of *B. pertussis* vaccine when the shock-inducing dose was given on 12th to 14th day rather than on 21st to 24th day. Our study confirmed their finding.

In general, intensity of shock after sensitization with bovine gamma globulin was not so severe as that produced by egg albumin, as reported by others(2,3). Sensitization with bovine gamma globulin, however, was suitable for our study as it permitted collection of lymph for 20 to 30 minutes after shock. The observation that there is a rough parallelism between degree of clinical shock and increased quantity of histamine suggests strongly that histamine plays an important role in production of anaphylactic shock in the rat. Undoubtedly other chemical mediators play a role.

Our efforts to deplete animals of histamine by preshock treatment with polymyxin B sulfate, roentgen rays, and distilled water can be considered as only preliminary. Although we followed the plan of administration of Sanyal and West(4) in giving polymyxin B sulfate, histamine was present in control lymph, and also was released after shock-inducing dose. The amount, however, was somewhat less than might have been expected with the degree of clinical shock observed.

Rats irradiated with roentgen rays were too ill to permit correlation of degree of clinical shock and quantity of histamine in lymph. In one rat we observed increase in flow of lymph usually seen in shocked animals, though no histamine was found.

Values for histamine in control lymph of all sensitized but otherwise untreated rats are similar to values in blood plasma. This was first noted in studies of blood and lymph done in the same animal by direct assay. Studies of lymph and plasma done by extraction method were done on different animals. Values of 0.015 to 0.026 μg of histamine/ml of

TABLE II. Histamine Content of Intestinal Lymph after Anaphylactic Shock Following Administration of Polymyxin B Sulfate, Roentgen Rays and Distilled Water.*

Pretreatment	Degree of shock	Specimen, min.	Histamine, μg/ml (expressed as base)
Polymyxin B	Good	Control	.03
		6	.24
		12	.05
		30	.05
"	Mild to none (shocked on 8th day)	Control	.03
		6	.00
		12	.12
		30	.01
"	Moderate	Control	.01
		6	.30
		12	.00
		30	.06
X-ray irradiation, 1 animal	None	Control†	.00
		6	"
		12	"
		30	"
" irradiation	"	Control	"
		6	"
		12	"
		30	"
Distilled water	Good	Control	"
		6	.80
		12	‡
		30	0
"	"	Control	.06
		6	.45
		12	.10
		30	.08

* Each histamine study represents lymph pooled from 2 animals.

† Histamine determined in this group of specimens by direct assay. Extraction procedure used in all others.

‡ Not enough to measure.

plasma are similar to values of 0 to 0.02 μg of histamine/ml in lymph.

Conclusions. 1) Bovine γ globulin is a satisfactory antigen for sensitizing rats when given intraperitoneally simultaneously with *Bordetella pertussis* vaccine. Intestinal lymph of animals so sensitized contains increased amount of histamine when collected 6 minutes after intravenous injection of shocking dose. The amount is roughly proportional to degree of shock. Rate of flow of lymph increases 8 to 25 times during the 12 minutes immediately after administration of shocking dose. 2) Lymph from mesenteric lymphatic system contains 0 to 0.02 μg histamine/ml, an amount similar to that in plasma. 3) A limited number of studies suggest that rats treated with polymyxin B sulfate and distilled water before shock were only partly depleted of histamine.

The author acknowledges encouragement and advice of Dr. C. F. Code during the study in his laboratory and also the aid of Dr. M. M. D. Williams, Section of Biophysics.

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Received April 19, 1960. P.S.E.B.M., 1960, v104.

Serum Folic Acid Activity by Improved *L. casei* Assay.*† (25900)

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Serum folic acid activity by microbiological assay with *Lactobacillus casei* has recently been proposed as a diagnostic test for folic acid deficiency(1,2,3). Levels less than 7.5 mμg/ml were reported indicative of the deficiency condition. Although folic acid activity of whole blood, plasma and serum had been extensively investigated by a variety of microbiological assays it had not previously been recommended as a means of assessing folic acid nutrition because activity levels in normals often were zero(4-14) and because the folic acid active compounds normally present in blood were presumably not folic acid or folinic acid(15). Nevertheless, because clinical folic acid deficiency is probably more common than generally appreciated and because laboratory means of assessing it are few(16), we undertook to evaluate the test proposed by Herbert *et al.*(1,3) and Baker *et al.*(2). In so doing, we found the assay they proposed lacked sensitivity at the lower levels which were of critical clinical importance. We wish to describe herein several improvements of the *L. casei* assay which greatly increase its sensitivity and accuracy and our experience with it in normal and folic acid deficient subjects. We found that levels in folic acid deficient subjects overlapped those of normals to such an extent that the assay had no clinical usefulness as a diagnostic test.

Materials and methods. Maintenance of stock culture. Lactobacillus casei ATCC 7469 was used in these studies, grown on the liver-tryptone agar of Nymon and Gortner(17) and maintained according to the scheme shown in Fig. 1. Three agar stabs, designated as monthly stabs, were prepared from the original culture. One monthly stab was used to prepare 4 weekly stabs, and the 2 remaining monthly stabs refrigerated until used. Each week 5 daily stabs were prepared from a weekly stab and the latter discarded. The remaining weekly stabs were refrigerated until used. A liquid broth inoculum was prepared each day from a daily stab, and the latter discarded. At end of month an unused monthly was employed to make 3 new monthly stabs and the scheme repeated. Spares were kept several months to be used in case of contamination in subcultures. *Inoculum preparation.* For liquid broth inoculum, 10 ml of skim milk medium previously described was used(18). A transfer from the daily agar stab was allowed to incubate at 37°C overnight (16-18 hours). 0.2 ml of uniformly suspended broth culture was transferred to a tube containing 6 ml of single strength assay medium to which 200 $\mu\mu$g folic acid had been added. This was allowed to incubate at 37°C for about 6 hours until O.D. of 0.1 to 0.12 was obtained in Coleman Jr. spectrophotometer at 660 mμ. The culture

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* Supported by grants from Nat. Inst. Arth. & Metab. Dis., U.S.P.H.S., Div. Cancer Control & Research, N.Y.C. Dept. Health, & R. E. Goldberg Memorial for Cancer Research.

† We are grateful to Drs. Lawrence B. Slobody, Martin L. Stone, Kenneth R. Crispell, for encouragement and permission to study their patients, as well as Drs. L. R. Wasserman, Mt. Sinai, M. S. Bruno, Knickerbocker, L. Thomas and L. Weiner, Bellevue, T. S. Spaet and V. Herbert, Montefiore, and A. Marcus, Man. V. A. Hospitals; J. M. Herrero for technical, and Drs. R. Feldman and P. A. Haddad for clinical assistance.

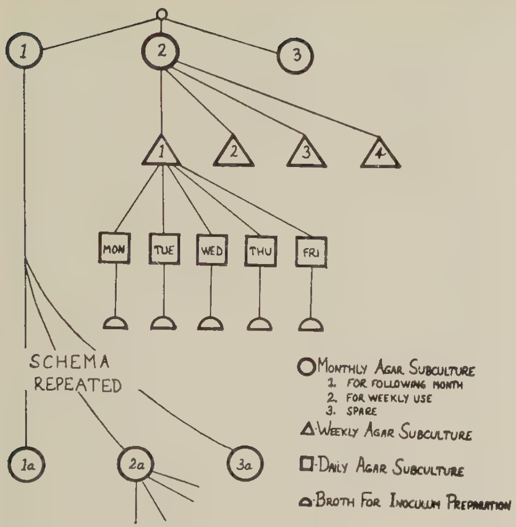

FIG. 1. Scheme for maintenance of *L. casei* on agar to insure culture stability.

was then centrifuged at 3000 rpm for 6 minutes, the supernatant discarded, and the bacteria resuspended in 6 ml sterile saline. A suitable aliquot was then diluted 10-fold with sterile saline. One drop of the latter was used to inoculate each assay tube. *Preparation of samples.* Venous blood was obtained from subjects in preprandial state and allowed to clot. Serum separated, was stored at –20°C until used. Serum was diluted 1:100 with phosphate-ascorbic acid buffer of Toennies *et al.*(19), incubated at 37°C for 90 minutes and autoclaved for 2½ minutes at 15 lb. pressure to precipitate protein. The clear supernatant when added to the assay tubes in amounts of 0.2, 0.5 and 0.8 ml provided dilutions of original serum of 1:500, 1:200 and 1:125 respectively. This compared with 1:20, 1:10 and 1:5 dilutions of original serum added to assay tubes by Baker *et al.*(2). *Assay medium and procedure.* The modified medium of Toennies *et al.*(19,20) was used. Assay was carried out in a total of 2 ml of liquid. This was found to increase its sensitivity. 0.2, 0.5, and 0.8 ml of serum sample prepared as above and sufficient distilled water to make 1 ml were added to duplicate 13 x 100 mm test tubes containing 1 ml of medium. For standard curve, folic acid was used at levels of 30, 50, 70, 100, 150, 200, 250, 300, 400 and 500 μμg per tube. Tubes were covered with aluminum caps, autoclaved at 15 lb. pressure for 2½ minutes, inoculated after cooling and allowed to incubate at 37°C for 16 hours. Turbidity was then read in a Coleman Jr. spectrophotometer at 660 mμ. The standard curve was plotted on semilogarithmic paper. *Clinical material.* Subjects classified as normal included laboratory personnel and clinic patients who showed no signs of malnutrition and had normal hemograms. Subjects classified as folic acid deficient had macrocytic anemia, various degrees of megaloblastic erythroid and myeloid changes in their bone marrow, and made a classical reticulocyte, erythrocyte and hemoglobin response to less than 0.8 mg of folic acid per day(21,22). All folic acid deficient patients excreted significantly increased amounts of urinary formiminoglutamic acid after a 72 hour 15 g L-histidine monohydrochloride metabolic load (23).

Results. Comparison of standard curves obtained using the method described here with that of Toennies *et al.*(19,20) and that of Baker *et al.*(2) are shown in Fig. 2. The greatest sensitivity was obtained with our method. The straight line portion of the curve extends from 30 to 500 μμg per tube. The assay method of Baker *et al.*(2) is least sensitive because the usable portion of the curve extends from 300 to 3000 μμg per tube.

Serum levels. Serum folic acid activity determined by our method in normal adults,

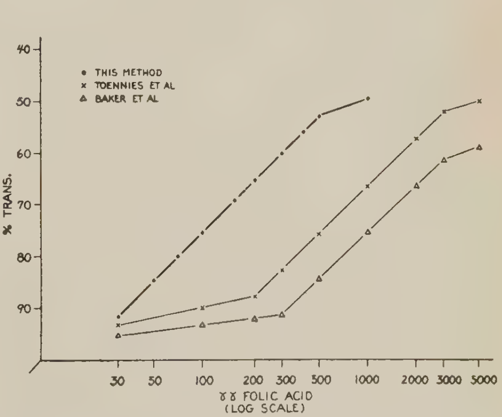

FIG. 2. Comparison of standard curves obtained by this method with that of Toennies *et al.* and Baker *et al.* Folic acid concentration expressed in μμg/assay tube; vol/tube our method, 2 ml; Toennies *et al.*, 6 ml; Baker *et al.*, 10 ml.

TABLE I. Serum Folic Acid Activity in Normal and Folic Acid Deficient Subjects Determined by Improved *L. casei* Assay.

	No. of subjects	mμg/ml Mean and S.D.*	Range
Adults:			
Normal	38	15.9 ± 9.5	4.0- 45.6
Folic acid deficient	30	9.9 ± 6.4	2.4- 31.5
Pregnant women:			
Normal	10	17.3 ± 6.5	9.5- 28.3
Folic acid deficient	10	10.2 ± 5.4	3.8- 22.8
Normal infants and children (5 wk to 3 yr)	40	27.5 ±33.8	2.7-201.2

* S.D. = stand. dev.

normal pregnant women in the last trimester of pregnancy, and normal infants from 5 weeks to 3 years of age is shown in Table I. Range of values found in each group was wide, for example, varying from 4.0 to 45.6 mμg/ml in normal adults. Mean level for normal pregnant women in the last trimester did not differ significantly from normal adults. The greatest variation occurred in normal infants, 5 weeks to 3 years of age, in whom levels from 2.7 to 201.2 mμg/ml were found. The mean level in this age group tended to be higher than in the other 2 groups.

In folic acid deficient adults and pregnant women mean levels of 9.9 and 10.2 mμg/ml respectively were moderately lower than mean value in corresponding normal subjects. However, the variation in serum folic acid activity in these deficient individuals was as great as in normals, 2.4 to 31.5 mμg/ml and 3.8 to 22.8 mμg/ml respectively.

Discussion. The improved assay method for serum folic acid activity described here has several advantages over previously described procedures. Maintenance of the culture in the prescribed manner on agar stabilizes growth characteristics of the organism so that consistently good standard curves are obtained. When the stock culture is maintained through broth transfer as recommended by Baker *et al.*(2), the organism eventually loses its folic acid requirement and considerable growth occurs in the growth control tubes containing no folic acid. The further modification of reducing the assay culture volume from 10(2) to 2 ml, permits extension of sensitivity from 300 μμg(2) to 30 μμg/tube. This makes possible more accurate assessment of serum folic acid activity in the low ranges. It is here that precise measurement is important to evaluate the possible clinical significance of this serum determination. The 10-fold increase in sensitivity also allows performance of an accurate assay on 0.1 ml of serum. This micromethod modification is of practical clinical advantage when larger amounts of serum are not obtainable.

Drs. Usdin and Toennies assayed serum of laboratory personnel at the Inst. of Cancer Research, Philadelphia, Pa., for folic acid activity with their *L. casei* method and have kindly allowed us to quote their unpublished results. These workers found serum folic activity to range from 2.65 to 10.6 mμg/ml with a mean of 5.49 and a standard deviation of ± 2.42 mμg/ml. These results are in agreement with our experience.

All the above results are contrary to those reported by Herbert *et al.*(1,3) and Baker *et al.*(2) in which a serum folic acid activity level below 7.5 mμg/ml by *L. casei* was *consistently* associated with clinical folic acid deficiency. Furthermore, the *wide overlap* of serum folic acid activity among normal subjects and folic acid deficient patients found in our studies makes this determination of little value as an index of folic acid deficiency.

Baker *et al.*(10,12,14) using a thermophile, *Bacillus coagulans,* assay, which presumably measures the same compounds as *L. casei*(2), found no correlation between serum folic acid activity and anemia in pregnant women. More recently, Baker *et al.*(24) reported serum folic acid activity by *L. casei* assay of *normal* pregnant women to range from 1 to 100 mμg/ml. Similarly the cord serum "activity" of *normal* newborns varied from 2.5 to 250 mμg/ml. These findings, more in conformity with our own results in normal adults, young infants and children, also cast doubt on the usefulness of this assay as a criterion of folic acid deficiency.

Summary. 1) An improved *L. casei* assay procedure for serum folic acid activity is described. Its range of usefulness over previ-

ous procedures is increased because of greater sensitivity, better accuracy in lower ranges, stability of test organism and micromethod applicability. 2) With this improved assay, the overlap of serum folic acid activity levels among normal subjects and folic acid deficient patients is so great that this determination has no value as an index of folic acid deficiency.

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Received May 4, 1960. P.S.E.B.M., 1960, v104.

Effects of DDD on Steroid Response and Blood Flow Through the Adrenal After ACTH.* (25901)

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The effects of ACTH in increasing corticosteroid secretion of the adrenal cortex and blood flow through the gland have been reported. That these 2 responses are not directly related does not seem to be commonly known. This paper records the dissociation of these phenomena and sets forth some quantitative measurements on vascular reaction. Administration of DDD (2,2-bis(parachlorophenyl)-1,1-dichloroethane) causes a decreased secretion of 17-hydroxycorticosteroids and the adrenal becomes refractory to stimulus from ACTH. However, in doses sufficient to prevent steroid formation, DDD does not affect vascular responses of the gland. These findings are considered important because some isomers of DDD are being used currently in human therapy(1).

Method. Data were derived from 185 mongrel dogs of both sexes. The preparation of DDD used was purchased commercially, and reportedly contains approximately 90% of the p,p′-isomer and 5-7% of the o,p′-isomer(2).

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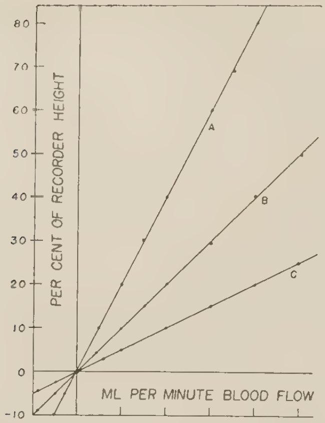
* This study was supported by U.S.P.H.S. grant.



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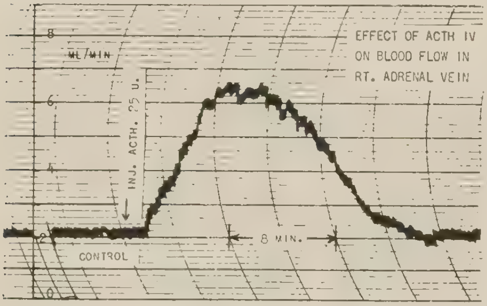

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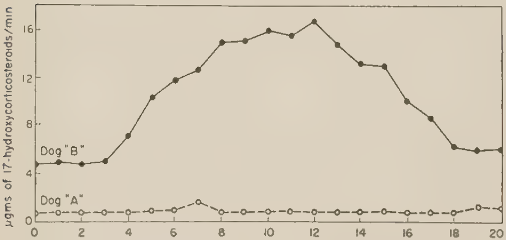

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FIG. 1. Calibrations of electromagnetic bloodflow meter, using 3 gain settings shown as plots A, B, and C. Gain setting for these studies approximated plot A.

FIG. 2. A typical adrenal blood flow response to administration of ACTH (25 units) after administration of sufficient DDD (condition A) to prohibit release of 17-hydroxycorticosteroids. This flow response to ACTH was not significantly different from that of dogs untreated by DDD, or from that of dogs treated by DDD in condition B as described.

FIG. 3. Graphic representation of 17-hydroxycorticosteroid response calculated as μg secreted per min. by adrenal of dogs after inj. of 25 units of Armour ACTH lyophilized powder. Dog "A" has been pretreated with 200 mg/kg of DDD once daily for 3 days. Note that low resting rate does not change after ACTH though there occurs a marked increase in blood flow (Fig. 1) and the gland is histologically normal. Dog "B" represents a similar response of a dog pretreated 3 days with 200 mg/kg of DDD and allowed 18 days to recover. This is a normal steroid response to ACTH despite the fact that adrenal shows definite histological changes characteristic of DDD.

In our experience the more potent o,p′- and m,p′-isomers do not have great advantage over commercial preparations in the dog(3). Eight male dogs weighing 10-15 kg were fed 200 mg/kg of DDD powder in gelatine capsules once daily for 3 days. They were then anesthetized with Surital† and the adrenal vein cannulated by the method of Hume and Nelson(4). On the day following operation the dogs were awake and in apparent good condition. They were again lightly anesthetized, the indwelling cannula flushed with heparin-saline solution, and the choker on the caval side of adrenal closed. All blood emerging from the cannula in the lumbo-adrenal vein was effluvent having passed through the adrenal. The dog was then heparinized and adrenal effluvent blood directed through an electromagnetic flow meter(5). To achieve the high flow sensitivity required in these studies, the flowmeter output was taken through a Brush DC amplifier into an Esterline-Angus 1500 ohm, 1 milliampere, recorder by using an impedance matching network. As a result of the modification, for 30 minutes on some occasions, the zero base line was known to shift from 0.1 to 3% of full scale allowing an error of less than 0.3 ml/min in actual measurement. For a flow of 6 ml/minute this entailed a possible error of 5% or less. Since all errors of zero shift were apparent by a zero check, this did not influence our measurements, but it was an inherent factor in the observations. All blood flow measurements reported here were calibrated and rechecked by an actual venous outflow technic through the cannula pickup, both for zero setting and flow responses, rather than chance employment of a synthetic flow system, alone. Representative samples of flowmeter calibrations are shown as 3 plots in Fig. 1. Experimental data were obtained in both DDD-fed and normal dogs by collection of one-minute flow samples of blood into centrifuge tubes which subsequently were analyzed for 17-hydroxycorticosteroids by a modification of the Nelson and Samuels(6) method. After about 5 minutes of control flow 25 units of Armour lyophylized ACTH powder (U.S.P.) was dissolved in saline and immediately injected into a peripheral vein. Collection of minute samples of blood was continued for the next 20 minutes as the graphic pattern of blood flow was recorded.

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† Surital kindly provided by Parke Davis and Co.

Results. Blood flow results in all 8 dogs were remarkably similar, *i.e.*, within 2-5 minutes blood flow markedly increased to attain a maximum within about 8-15 minutes to 260-400% of control blood flow, whereupon a gradual decline was observed requiring 5-10 minutes to attain pre-ACTH values, a total reaction time of 15 to 30 minutes. A representative tracing of this phenomenon (Dog-A) is shown in Fig. 2. Similar data were observed in control dogs. In contrast, 17-hydroxycorticosteroid secretory rate (0.6 μg/minute) continued unchanged at a basal rate throughout entire period of vascular response (Fig. 3). Thirty minutes after injection of ACTH the dog was killed, adrenals fixed in Zenker's solution, sectioned, stained and examined microscopically.

Five male dogs weighing 10-15 kg were fed 200 mg/kg of dry technical DDD powder in gelatine capsules once daily for 3 days only and 16-18 days later were treated as the dogs in the previous group. When 25 units of ACTH were injected a marked vascular response was obtained in each dog which simulated that of untreated dogs. Blood 17-hydroxycorticosteroids showed the usual normal resting secretory rate of 4.5 μg/minute which increased to 17 μg/minute at the end of 12 minutes. Return to essentially normal secretory rates of 7.1 μg/minute was effected in 20 minutes. This is a normal response as to time and magnitude when compared with 25 normal untreated dogs. Microscopic examination of adrenals from this group of dogs showed a uniform mild-early-atrophic change, consisting of a slight lymphocytic infiltration of the outer layers of the zona fasciculata together with an accumulation of lipids in large vacuoles of the parenchymal cells which pushed the nuclei of many cells to the periphery. Nuclei were small, somewhat pyknotic, and stained deeply basophilic. These are the very definite changes of early response of adrenal cortex to this compound.

Discussion. The foregoing shows that 3 days of administration of DDD to dogs resulted in the adrenals secreting 17-hydroxycorticosteroids at a very low rate, which did not increase with administration of ACTH, while the gland responded to ACTH with the usual increase of blood flow. These glands were histologically "normal" hemodynamically normal, but dysfunctional biochemically. Administration of 3 similar doses of DDD to dogs resulted in adrenals showing early histological change after 18 days on normal diet, yet these glands were secreting 17-hydroxycorticosteroids at a normal rate and responded in a normal fashion to ACTH. The anatomical change in these latter glands did not prevent the usual vascular response, suggesting that site of vascular response to ACTH may be in the vessels of the medulla or in the extracapsular vessels. It also would suggest that ACTH may have an extra "vasodilating component" separate from the "steroid releasing and/or steroid forming component." Such conclusions must be guarded, however, because the ACTH used was the relatively impure clinical preparation and doses were grossly pharmacologic. However, in separate experiments(unpublished), occasional increases up to 150% of control blood flow have been obtained with dog ACTH in doses of 0.005 unit. This would tend to minimize the dose and species problem. The vasodilator effect must be relatively specific for adrenal vasculature because a separate set of studies has been carried out by injecting standardized units of ACTH in the femoral artery of dogs and comparing response with the action of mecholyl. In the periphery, ACTH acts as a mild vasodilator, but its response is of low magnitude and low duration. Femoral flow increased less than 15% (115% of control) and lasted less than 20 seconds with the standardized dosage. Any detectable response in the dog limb is completed in less than 30 seconds. Intravenous injection has less effect on peripheral blood flow response than intra-arterial injection, as would be anticipated. On the other hand, standardized intravenous injection of epinephrine (1-20 μg) and mecholyl (.1-1.0 μg) produced little or no detectable flow response through the adrenal gland (mecholyl demonstrated a barely discernible flow increase at higher dosages of 10 μg). This readily apparent difference in vascular response from ACTH, epinephrine, and mecholyl in the adrenal vasculature as compared to the periphery deserves a more thorough study.

The histological finding that a "normal" gland, as revealed by microscopic examination of a hematoxylin and eosin preparation, does not secrete 17-hydroxycorticosteroids and is refractory to ACTH raises doubts as to the validity of many conclusions based on only microscopic appearances. It indicates that action of DDD has a latent period and emphasizes the uncertainty of judging the secretory status of the adrenal from microscopic examination. The finding that after 18 days a normal secretory pattern and response to ACTH is present in dog adrenals with cytological change is particularly interesting since Vilar and Tullner(7) have shown that after 4 days treatment with the o,p′-isomer histological changes are apparent, and secretion of corticosteroids is depressed, indicating that secretory capacity recovers while mild histological alteration continues. This delayed histological change from 3 doses of technical DDD has not heretofore been reported.

Conclusions. Technical DDD fed to dogs in 200 mg/kg doses once daily for 3 days resulted in histologically normal adrenal glands which responded to ACTH with the expected increase in blood flow, but demonstrated a low resting secretion rate of 17-hydroxycorticosteroids, which did not increase after administration of ACTH. Dogs fed a similar 3 doses and allowed to continue normal diet for 18 days showed adrenals with definite changes in the cortex which did respond to ACTH with the expected increase of steroid secretion but showed usual increase in blood flow. These data are interpreted to mean that the vasodilating factor of commercial ACTH is separate from steroid formation and/or releasing factor(s) and possibly the vasodilating factor may act on medullary or extracapsular vasculature. Capacity of the cortical parenchymal cells to form and/or release 17-hydroxycorticosteroids, as influenced by DDD, does not parallel histological changes observed in standard hematoxylin and eosin preparations.

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Received May 9, 1960. P.S.E.B.M., 1960, v104.

Interaction of Chlorpromazine with Strandin.* (25902)

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LeBlanc(1,2) has shown that chlorpromazine can destroy the mast cell, and has suggested that some of the effects of this drug may be due to release of histamine and serotonin from that cell. The mast cell is also considered to be the site of heparin production(3). During recent study concerned with metachromasy of brain ganglioside, strandin

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* Supported by grants from National Tay-Sachs Assn. and U.S.P.H.S.

(4), the similarity in structure between thiazine dyes and phenothiazine drugs was noted. Aggregation and micelle formation among metachromatic dyes is a well known phenomenon(5). Scholtan(6) studied aggregation among phenothiazine derivatives which contain alkylated ammonium groups, and concluded that these compounds behave as colloidal electrolytes in solution. Our preliminary experiments indicated that heparin and chrondroitin sulfate interact with some phenothiazine tranquilizers to form products of visible turbidity. In addition, strandin and the sulfated mucopolysaccharides (heparin and chondroitin sulfate) share certain common biochemical properties, such as inhibition of clotting mechanism(7,8), metachromasy(4,8) and interaction with basic proteins (9). It was considered of interest to determine whether a reaction occurs between phenothiazine tranquilizers and strandin.

Materials and methods. 1. The brain ganglioside, strandin, was extracted from the cortical gray matter of a case of Tay Sachs' disease, by the method of Folch, Lees and Sloane-Stanley(10). Tissue was maintained at –8°F in the deep freeze, prior to extraction. Strandin was analysed by the neuraminic acid method of Saifer and Gerstenfeld(11), assuming an average value of 30.5 mg of neuraminic acid per 100 mg of strandin(12). 2. Chlorpromazine and trifluoperazine (Stelazine) were obtained from Smith, Kline and French Laboratories, Philadelphia, Pa.† 3. Heparin (Sodium salt, 100 International Units per mg) was obtained from Connaught Medical Research Labs., Univ. of Toronto, Canada. 4. Chondroitin sulfate (CSA) was obtained from Pentex Inc., Kankakee, Ill. 5. Lysozyme (2× crystallized) was obtained from Worthington Biochemicals Corp., Freehold, N. J. 6. Toluidine Blue O (Total dye content of 94%) was obtained from Nat. Aniline Division, Allied Chemical and Dye Corp., N. Y. 7. All other chemicals used were of reagent grade. 8. All measurements of turbidity were made using the Bausch and Lomb Spectronic-20 spectrophotometer, set at a wavelength of 420 mμ. 9. Measurements of metachromasy were made with the same instrument using as an index of metachromasy the Optical Density Ratio (O.D.R.), which is equal to (O.D. 550/O.D. 635)(4). 10. Measurements of ultraviolet absorption spectra were made with Beckman DU Spectrophotometer. 11. Ultracentrifuge patterns were obtained with Spinco Ultracentrifuge-Model E, maintained at a velocity of 50,740 rpm, at 20°C. 12. Dialysis equilibrium was performed with "Visking" cellulose casings. Chlorpromazine remaining within the sac during period of dialysis was analysed by a colorimetric procedure described below.

Results. A) *Turbidimetric studies.* Phenothiazine derivatives form products of visible turbidity with heparin and chondroitin sulfate (CSA), quantitatively demonstrated in Fig. 1. Increased optical density values at 420 mμ, indicating increased turbidity, were used as the index of this interaction. Heparin reacts more readily than CSA, the latter requiring addition of a higher concentration of chlorpromazine before turbidity becomes optically apparent. Absorption at zero chlorpromazine concentration is due to the slight color of concentrated mucopolysaccharide solutions and not to turbidity. Qualitatively similar results were obtained with stelazine. When strandin (0.87%) is added to the heparin-chlorpromazine reaction product, there is a progressive decrease in turbidity, with increasing concentration of strandin, so that on addition of 2.61 mg (88 mg%) of strandin turbidity is reduced from 0.810 to 0.145, an 82.1% decrease (Fig. 2A). Upon titration of this system with chlorpromazine, turbidity reappears (Fig. 2B). Similar results were obtained on treating chondroitin sulfate-chlorpromazine with strandin, turbidity reappearing following titration with chlorpromazine. Results of both experiments were duplicated with stelazine. It has been demonstrated that strandin interacts with basic proteins(9); and metachromasy of strandin was found to be extremely sensitive to changes in the physicochemical environment(4). Turbidity of the

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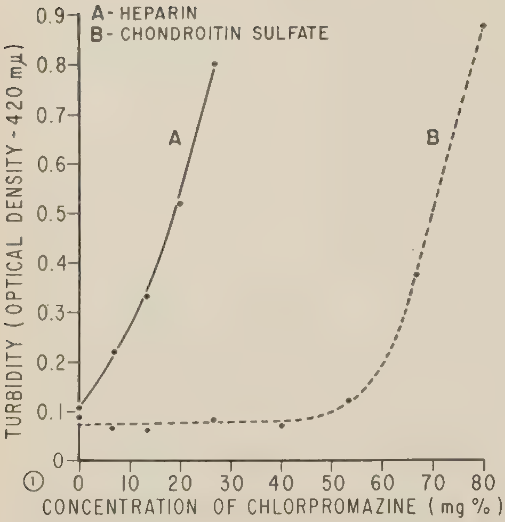
† We thank M. J. Ritchie, Med. Dept., Smith, Kline and French Labs., for providing a generous supply of these compounds.



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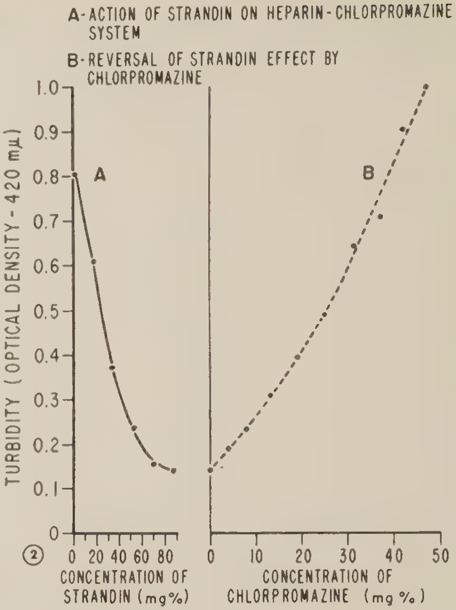

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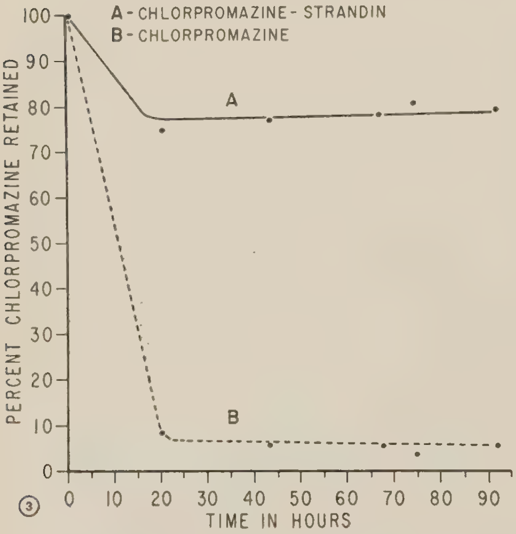

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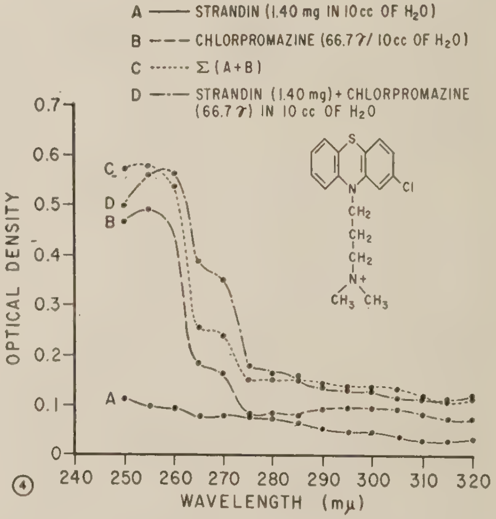

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FIG. 1. Interaction of chlorpromazine with heparin (A) and chondroitin sulfate (B). 2 ml of a 2% solution of acid mucopolysaccharide was reacted with amount of chlorpromazine indicated, in water. Vol constant at 3 ml. Temp. 26°C. pH change for heparin system 6.18 to 6.15 and for CSA 6.10 to 5.80.

FIG. 2. Reversible repression of heparin-chlorpromazine turbidity. A: 2 ml of 2% heparin plus 0.2 ml of 0.4% chlorpromazine plus indicated quantity of strandin (0.87%) in water. Vol constant at 3 ml. Temp. 26°C. pH change from 6.50 to 6.20. B: Titration of 2 ml of 2% heparin plus 0.2 ml of 0.4% chlorpromazine plus 0.3 ml of 0.87% strandin with chlorpromazine (0.4%) in water. Final vol 3.4 ml. Temp. 26°C. No pH change. (Final pH 6.20.)

FIG. 3. Equilibrium dialysis of chlorpromazine and chlorpromazine-strandin at 4.4°C. A: Chlorpromazine-strandin: 2 ml portions of 0.1% chlorpromazine plus 0.87% strandin, each dialysed against 30 ml of water. pH change within dialysis sacs was from 6.35 to 6.12. B: Chlorpromazine: 2 ml portions of 0.1% chlorpromazine; each dialysed against 30 ml water. pH change within dialysis sacs was from 5.42 to 6.51.

FIG. 4. Effect of strandin on ultraviolet absorption spectrum of chlorpromazine. A: 0.4 ml of 0.35% strandin (1.40 mg) in 10 ml of water. B: 10 ml of chlorpromazine (66.7 γ). C: Algebraic sum of absorption of A and B, Σ (A + B). D: 0.4 ml of 0.35% strandin (1.40 mg) plus 66.7 γ of chlorpromazine in 10 ml of water.

lysozyme-strandin interaction product is reduced by chlorpromazine. After titration of this chlorpromazine-treated system with strandin (0.4%) turbidity is partially regenerated. Metachromasy of the toluidine blue O-strandin system, as measured by the O.D.R. (O.D. 550/O.D. 635) is reduced by about 71.0% after titration with chlorpromazine.

B) *Equilibrium dialysis.* To obtain further proof of the proposed interaction between chlorpromazine and strandin, 5 (2 ml) solutions of chlorpromazine and chlorpromazine plus strandin were placed in dialysis sacs ("Visking" cellulose casing) in 30 ml of water. Initial concentration of chlorpromazine was 0.1% (2 mg/2 ml) and 0.87% strandin (17.4 mg/2 ml). Internal samples were analysed at intervals by the colorimetric method of Leach and Crimmin(13), employing ferric nitrate as the oxidizing agent and reading the resulting red solution at 530 mμ with the spectrophotometer. Results were computed from a standard curve as mg/l of chlorpromazine inside the dialysis sac, then converted to percent chlorpromazine retained by the sac. The series of experiments conducted without strandin shows a rapid loss of chlorpromazine from the internal phase of the system (Fig. 3B), so that only about 7.5% remains after the first 20 hours. At end of 92 hours, the internal phase has retained but 5.5% of original amount of chlorpromazine. In the system which contains strandin (3A), marked retention of chlorpromazine occurs. Thus in the first 20 hours, this system has retained 77.5% of the original amount of chlorpromazine, and this level of internal concentration is maintained throughout the remaining 72 hours of experiment, as indicated by analysis of the remaining 4 sacs.

C) *Effect of strandin on ultraviolet absorption spectrum of chlorpromazine.* Fig. 4A shows ultraviolet absorption of 0.4 ml of 0.35% strandin (1.40 mg) in 10 ml of water. At this concentration little absorption is demonstrable. Fig. 4B represents absorption spectrum of chlorpromazine (66.7 γ/10 ml). Fig. 4C demonstrates the algebraic sum of A (strandin) plus B (chlorpromazine) absorption. It is the theoretical absorption which should result from a mixture of the 2 substances in absence of interaction. The observed curve resulting from mixture of 0.4 cc of 0.35% strandin (1.40 mg) in 10 ml of water containing 66.7 γ of chlorpromazine is indicated in Fig. 4D. When compared with the theoretical curve (Fig. 4C), maximum optical density has shifted from 255 mμ to 258 mμ. In addition overall absorption in the range 257.5 mμ to 285 mμ is greater than that of the theoretical curve. Beyond 275 mμ the changes are small and do not appear to be of great significance. The shift in absorption maximum of stelazine is greater, shifting from 256 mμ to 266 mμ for the observed system (0.4 ml of 0.35% strandin (1.40 mg) in 10 ml of stelazine (100 γ/10 ml). There is a noticeable increase in absorption in the range of 262.5 mμ to 272.5 mμ, in the case of the observed as opposed to the theoretical curves. Beyond 272.5 mμ changes are small.

D) *Effect of chlorpromazine on ultracentrifuge pattern of strandin.* The following solutions were analysed in the ultracentrifuge (50,740 rpm and 20°C) using an analytical cell with a one degree prismatic window. (a) 0.44% strandin in water adjusted to pH 5.0 with 0.1N HCl, (b) 0.44% strandin in 0.5% chlorpromazine at pH 5.0. The results of this experiment are indicated in Fig. 5A and 5B. Upper portion of the diagrams represents the pattern of strandin alone, lower portion indicates the pattern resulting from action of chlorpromazine. Fig. 5A represents the pattern at commencement of experiment, and 5B the pattern after 64 minutes.

At beginning of the run, the strandin pattern shows 2 peaks (a phenomenon not found at pH 6.7); after 64 minutes only one peak of smaller size is noted. A single large peak is found in the chlorpromazine-strandin mixture at beginning of the run; after 64 minutes a peak of medium size is found preceded by a shoulder which appears to be coincident with that found in the upper pattern representing strandin alone. With a solution of 0.5% chlorpromazine in water, (pH 6.0), no peaks were observed. It seems probable that the medium-sized peak, shown by the strandin-chlorpromazine mixture (5B), represents a

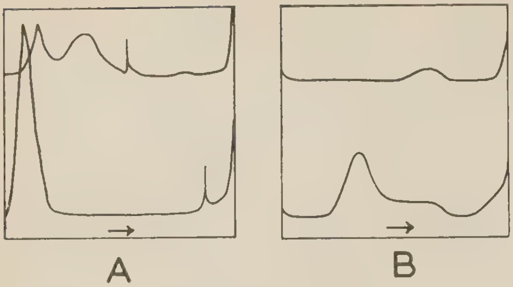

FIG. 5. Ultracentrifuge patterns of strandin (upper) and strandin plus chlorpromazine (lower), taken at commencement of run (5A) and after 64 min. (5B). Samples were spun at 50,740 rpm at 20°C. Strandin solution consists of 0.44% of the ganglioside in water at pH 5. Mixture consists of 0.44% strandin in 0.5% chlorpromazine at pH 5. The arrow above the abscissa indicates the direction of movement of sedimenting boundary.

slow moving complex of strandin with chlorpromazine.

Discussion. Chlorpromazine functions as an inhibitor of a number of enzyme systems. Thus it suppresses oxidative phosphorylation (14), cytochrome oxidase(14) ATPase(14) and D-amino acid oxidase(15). Yagi has presented evidence indicating that complex formation occurs between chlorpromazine and flavine adenine dinucleotide(16). LeBlanc (1,2) has indicated that chlorpromazine can destroy the mast cell. The present studies suggest that chlorpromazine and stelazine can interact with heparin and CSA to form products of visible turbidity.

Little is known concerning the physiological function of strandin, aside from its inhibition of the blood clotting mechanism(8) and influenza virus hemagglutination(17). It reacts with basic dyes(4) and basic proteins (9), most probably *via* the carboxyl groups of the neuraminic acid moiety of the molecule. It seems likely that this grouping is responsible for its interaction with chlorpromazine, the latter reacting *via* its positively charged nitrogen. This, however, is speculative, and further evidence will be required to ascertain the nature of the bond existing between these substances. Interaction of strandin with chlorpromazine and stelazine may have a bearing on mode of pharmacological activity of the phenothiazine derivatives. It is thought that these drugs primarily act on the midbrain (18). Since concentration of strandin is greater in the gray matter of the cortex, the presence of strandin may serve to protect the cells of the cortex from the drug.

Summary. 1) Chlorpromazine and stelazine interact with heparin and CSA to form products of visible turbidity. This turbidity is reduced by strandin, and reappears on re-addition of phenothiazine. 2) Chlorpromazine reduces turbidity of the strandin-lysozyme interaction product, turbidity of which may be regenerated by subsequent addition of strandin. It also destroys metachromasy of the toluidine blue O-strandin system. 3) Equilibrium dialysis of chlorpromazine solutions and chlorpromazine-strandin solutions indicates good retention of chlorpromazine in the system containing strandin. 4) Strandin alters U.V. absorption spectrum of chlorpromazine and, more markedly, that of stelazine. 5) Ultracentrifuge studies indicate presence of slow moving peak in the strandin-chlorpromazine system, which may represent strandin-chlorpromazine interaction product.

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Received May 9, 1960. P.S.E.B.M., 1960, v104.

Microbial Resistance to Penicillin as Related to Penicillinase or Penicillin Acylase Activity. (25903)

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Presence of enzyme penicillinase that specifically destroys penicillin activity by degradation to inactive penicilloic acid, offers a ready explanation for resistance of certain microorganisms to this antibiotic. With recent discovery(1) that many microorganisms are able to cleave the acyl group in penicillin G, to yield the relatively inactive moiety 6-aminopenicillanic acid (6-APA), still another mechanism for penicillin resistance became apparent. A number of bacteria are reported here to relate their resistance to penicillin to either penicillinase or penicillin acylase activity.

Results. It is obvious that 6-APA possesses definite antibacterial properties, but to a much less degree than penicillin G (Table I). Comparative values expressed as minimum inhibitory concentration (MIC) in μg/ml in Table I indicate that 6-APA is 4166-fold to 390-fold less active than penicillin G when tested *in vitro* against a series of Gram positive bacterial genera. Although the differences against the Gram negative bacteria are not so striking, 6-APA is 2-fold to 10-fold less active than penicillin G. Differences in activity similar to this were obtained *in vivo*. At concentrations of 200 mg/kg 6-APA failed to protect mice against experimental *Staphylococcus aureus* infections, and levels of 50 mg/kg against *Streptococcus pyogenes* infections offered no protection. In contrast, penicillins G and V were highly effective (Table I).

The relationship of MIC to penicillin, and presence or absence of penicillinase or penicillin acylase activity, for a number of different bacteria are shown in Table II. After an average MIC for penicillin was established, bacterial cells of each culture were examined for presence of penicillin acylase activity, *i.e.*, hydrolytic conversion of penicillin G to 6-APA, by the chromatographic procedure described by Batchelor(2). In addition, supernates were examined by several methods for evidence of biological inactivation of penicillin. In one system sterile filtrates of 24 hour cultures were achieved by passage through sin-

TABLE I. Comparison of Activity of 6-Aminopenicillanic Acid and Penicillin G.

I. *In Vitro*	MIC, μg/ml*	
Microorganism	6-Aminopenicillanic acid	Penicillin G
Staphylococcus aureus	125	.03
Streptococcus pyogenes	15.6	.006
” *faecalis*	250	.16
Diplococcus pneumoniae	250	.0078
Erysipelothrix rhusiopathiae	125	.08
Corynebacterium diphtheriae	31.2	.09
Bacillus subtilis	62.5	.02
” *cereus*	125	.04
Aerobacter aerogenes	62.5	25
Escherichia coli	6.25	25
Proteus vulgaris	250	25
Pseudomonas aeruginosa	250	100
Salmonella typhosa	125	12.5
” *pullorum*	62.5	12.5
Klebsiella pneumoniae	62.5	12.5
Neisseria gonorrheae	1.9	.19
Shigella sonnei	125	50
Brucella bronchiseptica	125	100

II. *In Vivo*	PD_{50}, mg/kg			
	6-Aminopenicillanic acid		Penicillin†	
Infection in mice‡	Oral	I.V.	Oral	I.V.
Staphylococcus aureus	>200	>200	1.5	.78
Streptococcus pyogenes	>50	>50	.9	.6

* 2-fold 10 tube serial dilution technic—MIC read after 20 hr incubation at 37°C.

† Penicillin V used orally; penicillin G used intravenously.

‡ Intraper. infection—treatment started ½ hr later—single dose. PD_{50} calculated after 96 hr.

tered glass filters. *Streptococcus pyogenes* ATCC 8668, highly sensitive to penicillin and free from penicillin acylase activity, was suspended in this filtrate. This served as inoculum in a 2-fold serial dilution test with a penicillin concentration of 100 μg/ml in the first tube. If no penicillin-inactivating enzymes were present in the supernatant broth, the resulting MIC of penicillin for this organism was 0.006 μg/ml. If such enzymes were present, the resulting MIC would be considerably higher than 0.006 μg/ml.

A second method made use of a modified Gots technic(3). An inhibitory concentration of penicillin (0.312 μg/ml) was incorporated into brain heart agar plate with *Staph. aureus* (penicillin sensitive). If a sterile paper disc moistened with a broth containing penicillin-inactivating enzymes is placed on the surface of such a plate, enzyme activity diffuses into the medium, penicillin is inactivated, and a halo zone of growth occurs. It is probable that both penicillinase and penicillin acylase activity in a broth would appear as positive by these technics. However, 18-24 hour broths from cultures known to contain penicillin acylase activity as determined by the chromatographic procedure, failed to produce a positive test in either the broth or plates technics. As an explanation, additional studies indicated that under our experimental conditions, penicillin acylase activity is more intracellular than that of penicillinase. Hence, such broth filtrates as above contained little if any, penicillin acylase activity. It is therefore assumed that inactivation of penicillin resulted from the action of penicillinase. In addition, probably the actual quantitative amount of penicillin acylase activity present is important.

As indicated for bacteria presented in section I of Table II, penicillinase activity was not detected. However, all contained penicillin acylase activity. Therefore, resistance to penicillin in these cultures can be explained by conversion of the highly-active penicillin to the considerably less active 6APA. As shown for these bacteria in section II of Table II, penicillin acylase activity was not detected. However, in each instance penicillinase activity was present. Penicillinase production by Gram negative bacteria including the representative genera in Table II has been reported by a number of authors(4,5).

TABLE II. Penicillinase and Penicillin Acylase Profiles of Microorganisms Resistant to Penicillin.

Microorganism	No. of strains	Avg MIC, μg/ml, penicillin	Presence of Penicillinase activity	 Penicillin acylase activity
Section I				
Aerobacter aerogenes	3	25	Neg	Pos
Escherichia coli	5	25-50	"	"
Proteus vulgaris	4	25-50	"	"
Pseudomonas aeruginosa	2	25-50	"	"
Pseudomonas phaseolicola	1	100	"	"
Section II				
Escherichia coli	5	25-50	Pos	Neg
Pseudomonas aeruginosa	2	25-50	"	"
Aerobacter aerogenes	1	25	"	"
Section III				
Proteus vulgaris	3	25	Neg	"
Escherichia coli	2	25-50	"	"
Pseudomonas aeruginosa	3	25-50	"	"
Section IV				
Staphylococcus aureus, penicillin resistant	1	>100	"	"
Staphylococcus aureus, clinical isolates, penicillin resistant	10	>100	Pos	"
Streptococcus pyogenes	2	.006	Neg	"
Staphylococcus aureus	3	.0156	"	"
Diplococcus pneumoniae	3	.0078	"	"

Although one might suspect the presence of both penicillinase and penicillin acylase activities in the same microorganism, such was not detected in any of those listed. Since there seems to be no logical explanation that would negate such a possibility, our studies are continuing.

The fact that still another metabolic mechanism or mechanisms in addition to that of penicillinase and penicillin acylase activity, confers resistance to penicillin is shown by the bacteria in section III of Table II. Although resistant to large concentrations of penicillin, neither penicillinase nor penicillin acylase activity was detected in these microorganisms. In contrast, the usual Gram positive bacteria have a similar profile, neither penicillinase nor penicillin acylase activity. However, these microorganisms are sensitive to low concentrations of penicillin (section IV, Table II).

It is apparent that presence or absence of penicillinase and penicillin acylase activity is not strictly related to MIC values for penicillin. These values remain constant for different genera listed in categories I, II, and III, and appear independent of presence or absence of penicillinase or penicillin acylase activity. Probably the final effect of penicillin (as indicated by the MIC value), penicillinase and penicillin acylase actions depends upon the killing rate of penicillin and enzyme production rate. The latter must not be rapid enough and is probably not produced in sufficient amount to interfere drastically with penicillin action.

Penicillin resistant *Staph. aureus,* IV of Table II, is resistant to penicillin and was obtained by selection in the laboratory technic of serial transfer in presence of gradually-increasing concentrations of penicillin. Such strains usually differ from penicillin-resistant clinical isolates because they fail to show the presence of penicillinase. Penicillin-resistant strains obtained from patients do contain penicillinase(6). This is shown in IV, Table II. The presence of penicillin acylase activity was anticipated in such laboratory-developed penicillin-resistant strains. Such activity has not been detected. Some mechanism other than penicillinase or penicillin acylase activity is indicated.

It is of interest to note the strain specificity relevant to the source of penicillinase or penicillin acylase activity. Various strains of *Escherichia coli* and *Pseudomonas* appear in 3 of the classifications in Table II; *Aerobacter aerogenes* appears in 2, etc.

Summary. 1. The penicillin moiety, 6-aminopenicillanic acid, has antibacterial activity but is considerably less active than penicillin. 2. Resistance to penicillin in one group of bacteria could be explained logically by presence of penicillinase activity, *i.e.,* conversion of penicillin to the inactive penicilloic acid. 3. Resistance to penicillin in a second group of bacteria could be explained logically by presence of penicillin acylase activity, *i.e.,* conversion of penicillin to the relatively inactive 6-aminopenicillanic acid. 4. In a third group of bacteria resistant to penicillin, neither penicillinase nor penicillin acylase activity could be detected. Still another metabolic mechanism other than penicillinase or penicillin acylase activity must be present in selected bacteria.

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Received May 11, 1960. P.S.E.B.M., 1960, v104.

Antagonism of Phospholine (Echothiophate) Iodide* by Certain Quaternary Oximes. (25904)

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It was previously reported that 2 oximes bearing quaternary nitrogen atoms were highly effective against Phospholine Iodide toxicity when both were injected intraperitoneally in the mouse(1). These compounds were Protopam Iodide (pralidoxime iodide, 2-PAM, 2-pyridine aldoxime methiodide) and TMB4 (1, 1′-trimethylene-bis(4-formylpyridinium bromide)dioxime). Phospholine is under investigation in treatment of myasthenia gravis(2) and systemic actions have been observed during its use as a topical miotic in glaucoma(3). Hence it appeared important to investigate its relationship to the oximes further and to compare antidotal activity of the oximes by oral and parenteral administration. TMB4 and Protopam are, of course, also of interest in connection with poisoning due to anticholinesterases which may be used as pesticides or chemical warfare agents(4,5).

Methods. Acute lethal doses were estimated in white male mice of CF1 strain and 25 ± 5 g in weight. Concentrations were such that total solution administered fell between 0.1 and 1.5 cc. Doses were spaced in most cases by a factor of 1.41 with 5 animals at each dose level. Deaths were counted at the end of 24 hours but actually 92% of all deaths occurred within one hour and 83% within 15 minutes. Symptoms came on within a few minutes and had entirely disappeared in animals surviving 24 hours. The LD_{50} and its standard error was calculated by the method of Miller and Tainter(6). The experimental design consisted in estimation of the LD_{50} of Phospholine with and without administration of a selected dose of antidote. Phospholine was always given intraperitoneally. Intraperitoneal injection of an oxime was given within a few seconds of Phospholine. Oral administration of an oxime was by stomach tube at intervals of 30 minutes to 24 hours before Phospholine. TMB4 was synthesized by the method of Poziomek and coworkers(7) and appeared to be identical with a sample kindly supplied by Dr. B. J. Jandorf of the U.S. Army Chemical Warfare Labs. Protopam Iodide was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis. and recrystallized from aqueous isopropanol: m.p. 220—2°C decomp.; E_M at 294 mmu, 12,500. Protopam Chloride was prepared by passing a saturated solution of Protopam Iodide through a column of anion exchange resin, evaporating the effluent and recrystallizing from a 1:1 mixture of absolute methanol and ethanol; m.p. 229—30°C decomp.; E_M at 294 mmu, 12,600; analysis, theory N-16.23%, Cl-20.53%, found N-15.94%, Cl-20.40%.

Results. The LD_{50} of Phospholine after oral or intraperitoneal administration of oximes is given in Table I. Slopes for dosage mortality curves in units of logarithms and probits are given for each LD_{50}. The control LD_{50} for Phospholine Iodide without oximes was taken as 0.229 ± 0.0057 which is a weighted mean of 9 determinations. Range was from 0.200 ± 0.013 to 0.256 ± 0.019. Slopes ranged from 8.5 to 16.1 with a mean of 12.0.

Lethal doses of Protopam Iodide and Chloride were respectively 235 ± 32 mg/kg (.91 ± .12 millimol/kg) and 205 ± 14 mg/kg (1.19, ± .081 millimols/kg) by intraperitoneal injection and ca. 4000 mg/kg (15.2 millimols/kg) and 4100 ± 890 mg/kg (23.7 ± 5.1 millimols/kg) by oral administration. The oral LD_{50} of Protopam Iodide could only be approximated because of poor water solubility. As antidotes, the 2 compounds administered intraperitoneally at .58 millimol/kg raised the LD_{50} of Phospholine to 12.6 ± 1.4 for the Chloride and 12.8 ± 2.1 for the Iodide. In the same way the LD_{50} of Phospho- 60 minutes after an oral dose of 4.6 millimols/kg of the Chloride was 5.4 ± .86

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* Diethoxyphosphinylthiocholine iodide.

TABLE I. Effect of Oximes on Lethal Dose of Phospholine Iodide in the Mouse.

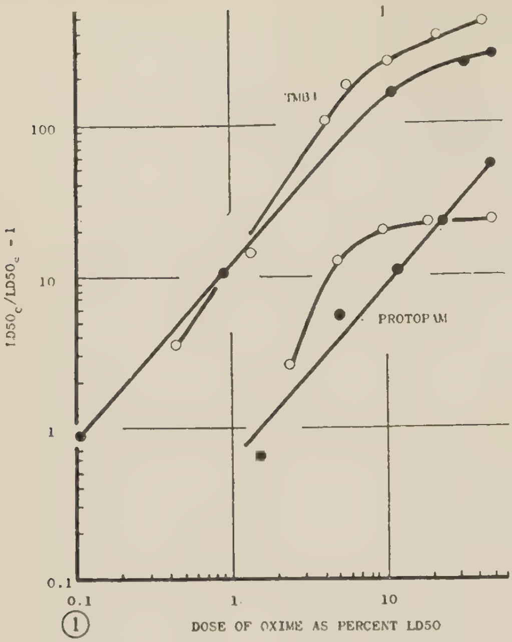
Oxime and route of admin.	Dose of oxime in mg/kg	Time between admin. of oxime and Phospholine	LD_{50} of Phospholine ± stand. error	Slope*
TMB4, intraper.	.1	0	.43 ± .057	11.2
	1		2.70 ± .38	7.5
	12		36 ± 4.9	7.7
	36		55 ±11	5.5
	54		64 ± 4.7	14.1
" , oral	20	30 min.	1.06 ± .10	11.2
	62.5		3.5 ± .45	8.1
	200		23.6 ± 2.2	11.2
	250		39.5 ± 6.2	5.5
	500		58 ± 9.7	5.2
	1000		85 ±12	7.5
	2000		103 ±14	7.6
	20	60 min.	.74 ± .12	6.7
	2000		127 ±20	5.5
	20	120 min.	.92 ± .11	8.7
	2000		135 ±17	8.2
	20	240 min.	.46 ± .060	11.2
	2000		90 ±14	5.4
	20	24 hr	.217± .025	12.7
	2000		1.64 ± .23	7.4
Protopam chloride, intraper.	3	0	.38 ± .026	16.1
	10		1.47 ± .19	11.8
	25		2.70 ± .20	27.0
	50		5.3 ± .83	5.5
	100		12.6 ± 1.4	7.7
" " , oral	100	30 min.	1.00 ± .14	10.5
	800		3.2 ± .57	8.3
	100	60 min.	.84 ± .11	10.9
	200		3.1 ± .81	4.1
	400		4.8 ± .75	5.6
	800		5.4 ± .86	5.4
	2000		5.6 ± .38	15.4
	100	120 min.	.46 ± .063	10.6
	800		4.1 ± .63	9.6
	100	240 min.	.33 ± .055	6.3
	800		2.6 ± .32	8.3
	100	24 hr	.205± .033	6.6
	800		.221± .031	7.5

* Slope estimated graphically and expressed in units of log dose and probit mortality.

and of the Iodide 7.7 ± 1.1. It is felt that these 2 salts are pharmacologically equivalent. Therefore, all subsequent work was carried out with the Chloride since this would appear to be the preferable salt for clinical use due to its excellent solubility and avoidance of possible side reactions to iodide ion.

In Fig. 1 data for the effect of TMB4 and Protopam Chloride by intraperitoneal injection and for the same drugs 30 and 60 minutes respectively after oral administration on LD_{50} of Phospholine have been transformed and plotted on logarithmic scales. Doses of oxime expressed as percentage of lethal dose appear as abscissae and $\frac{LD_{50_C}}{LD_{50_S}} - 1$ as ordinates,[†] where LD_{50_C} and LD_{50_S} refer to LD_{50} of Phospholine with and without antidote respectively. As a practical matter, doses of antidotes were not carried beyond one-half of their LD_{50} at which level mortality did not occur from the oxime alone.

[†] (x-1) of Schild(8).



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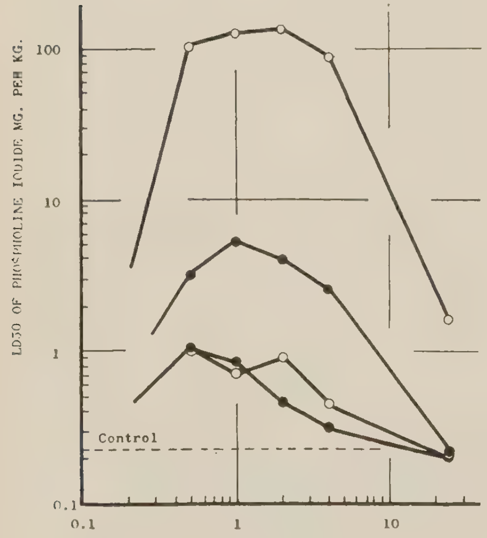

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FIG. 1. Relationship of log dose of oxime expressed as % LD_{50} and log $\left(\frac{LD_{50_C}}{LD_{50_S}} - 1\right)$ where LD_{50_C} and LD_{50_S} refer to lethal dose of Phospholine with and without oxime respectively. ○, oral administration of TMB4, 30 min., and Protopam, 60 min., before Phospholine. ●, intraper. inj. of TMB4 and Protopam.

FIG. 2. Relationship of LD_{50} of Phospholine to time interval between oral administration of oxime and estimation of LD_{50}. ○, TMB4 at 2000 mg/kg (upper graph) and 20 mg/kg (lower graph). ●, Protopam at 800 mg/kg (upper graph) and 100 mg/kg (lower graph).

TABLE II. Dose of Oximes Necessary to Raise the LD_{50} of Phospholine by a Factor of 10.

	Dose of oxime mg/kg	Dose of oxime % of LD_{50}	Therapeutic index*
Protopam chloride			
Oral	176	4.3	23
Intraper.	24	11.5	9
TMB4			
Oral	40	.88	114
Intraper.	.86	.83	120

* LD_{50}/ED_{50} where effective dose is that necessary to raise LD_{50} of Phospholine 10-fold.

The curves for TMB4 are linear over a considerable range of doses after which curvature appears. Oral and intraperitoneal plots are remarkably similar in shape and position so long as dose of oxime is expressed in terms of LD_{50}. Hence the relative activity of this drug by the 2 routes is approximately equal to the inverse ratio of its oral to intraperitoneal LD_{50} or 1 to 43. At 50% of LD_{50} of TMB4 the LD_{50} of Phospholine was raised by factors of 450 and 280 for oral and intraperitoneal administration respectively.

In the case of Protopam oral and intraperitoneal curves are not as much alike, the former showing a definite ceiling while the latter gives no sign of curvature up to one-half LD_{50}. Furthermore, the range of doses over which Protopam is effective is narrower than in the case of TMB4. At 50% of the LD_{50} of Protopam the LD_{50} of Phospholine was raised by factors of 23 and 50 for oral and intraperitoneal administration respectively.

Comparison of activities of the oximes in terms of percentages of LD_{50} which cause a 10-fold increase in the LD_{50} of Phospholine (graphical interpolation at 9 on the axis of ordinates of Fig. 1) is given in Table II. The reciprocals of these percentages multiplied by 100 are therapeutic indices at the arbitrarily selected activity level and are also given in the Table. Protopam has a better therapeutic index orally than intraperitoneally, while TMB4 is the same by either route. TMB4 has an index 5 times as great as Protopam by oral administration and 13 times by intraperitoneal injection.

Oral activities of the oximes were determined by injection of Phospholine after various intervals and in Fig. 2 is shown the relationship of time interval to LD_{50} of Phospholine. Doses of oximes were selected from the results in Fig. 1 as those that would be expected to show roughly maximum and minimum effects. Peak responses are not sharp and are spread between 30 and 120 minutes depending on dose level. Except for TMB4 at a high dose level, the effect of the oximes was completely dissipated after 24 hours. In comparing the data obtained by oral administration (Fig. 1), it should be understood that TMB4 was tested at a 30 minute interval while Protopam was at 60 minutes. In view of the results in Fig. 2 it is not felt that this discrepancy has any practical significance. The origin for the curves in Fig. 2 is uncertain. There would probably be some protective effect even if both Phospholine and oxime were given simultaneously since a certain amount of absorption of oxime would occur from the intestinal tract during the few minutes required for absorption of Phospholine from the peritoneal cavity.

Discussion. An extensive literature has developed on use of Protopam and TMB4 against poisoning by Sarin, TEPP, Parathion, isoflurophate and others. Here, the antidotal action of the oximes is of a limited order of magnitude unless atropine is also given. This is generally assumed to be due to the failure of the quaternary oximes to enter the central nervous system in contradistinction to the above group of cholinesterase inhibitors which do so. Phospholine appears to be the only phosphate type anticholinesterase bearing a quaternary nitrogen atom which is presently in clinical or industrial use and the marked effectiveness of the oximes without atropine presumably depends upon the fact that Phospholine also fails to cross the blood brain barrier.

Both oximes have potential value as antagonists to Phospholine and Protopam is already being used for this purpose. While TMB4 has the greater therapeutic index the activity of Protopam nevertheless would appear to be more than adequate for practical purposes.

It is of interest to consider the nature of the antagonism of Phospholine by quaternary oximes. The slopes in Table I show considerable variation which appears to be random with no systematic progression throughout any series. As would be expected, slopes for replicate assays of Phospholine alone are more consistent. While a rigorous statistical test does not seem justified because of the small number of animals used for each LD_{50}, there is probably no significant departure from parallelism among dosage mortality curves for Phospholine alone and when given with antagonists. This observation, together with the linearity over a considerable range of the curves relating $\log\left(\frac{LD_{50_C}}{LD_{50_S}}-1\right)$ and log dose of oxime (in units of LD_{50}), furnishes presumptive evidence of competitive antagonism(8). However, such a conclusion is perhaps hazardous when dealing with a system as complex as the whole animal and in view of the fact that tissue concentrations of the oximes may not be linearly related to dose. *In vitro* experiments have shown that both oximes reactivate the acetylcholinesterase of whole mouse blood which has been exposed to Phospholine and it has been assumed that this is the basis of the antidotal action *in vivo* (1). However, degree of reactivation of the enzyme possible in a given time falls slowly as the exposure period over which Phospholine is allowed to react increases (24% in 2 hours). Since the mice used in the present experiments died very promptly after a lethal dose was administered, it is reasonable to ignore this factor. It is sugestged that the antagonism of Phospholine by the oximes is competitive within the first hour or 2 during which Phospholine is probably acting as a whole molecule, and that a different relationship must obtain in later periods. It is not yet clear whether this is due to phosphorylation of the enzyme accompanied by splitting of the Phospholine molecule or to conversion of an enzyme—diethyl phosphate to monoethyl phosphate as suggested by the work of Cohen, *et al.*(9).

Summary. Protopam (pralidoxime) Chloride (or Iodide) and TMB4 (1, 1′-trimethylene-bis(4-formylpyridinium bromide) dioxime) when injected intraperitoneally in the mouse at 50% of LD_{50}, raise the intraperitoneal lethal dose of anticholinesterase, Phospholine (echothiophate) Iodide, by factors of 50 and 280 respectively. When the same oximes are given orally 30 to 60 minutes before Phospholine the intraperitoneal LD_{50} is increased by factors of 23 and 450. Graphs relating log dose of oxime and log dosage ratio of Phospholine are linear over a considerable range. The therapeutic indices for arbitrary increase of 10 times in LD_{50} of Phospholine are 23 and 9 for Protopam and 114 and 120 for TMB4 by oral and intraperitoneal administration respectively. The possible nature of the antagonism is discussed.

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Received May 12, 1960. P.S.E.B.M., 1960, v104.

Comparative Inhibition of Pyridoxal Kinase and Glutamic Acid Decarboxylase by Carbonyl Reagents.* (25905)

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γ-Aminobutyric acid (γABA) has been implicated as a transmission regulator in the central nervous system(1), though experimental evidence for such a role is ambiguous. Formation of this compound in brain from L-glutamic acid is catalyzed by a decarboxylase which requires pyridoxal phosphate as coenzyme(2). Carbonyl reagents are known to inhibit most pyridoxal phosphate enzymes through formation of a coenzymatically inactive derivative with the cofactor(3). Many of these reagents are now recognized as stimulants of the central nervous system ("psychic energizers") which in sufficient excess, produce epileptiform seizures that may be alleviated by administration of Vit. B_6(4). Localization of glutamic decarboxylase in brain(2), together with an observed reduction in levels of γABA following treatment by carbonyl reagents(5) have been interpreted to mean that both analeptic and convulsive activities of active carbonyl reagents may result from interference with production of this inhibitor of neuronal transmission by the decarboxylase (6). However, pyridoxal kinase catalyzes formation of pyridoxal phosphate from Vit. B_6 and has been shown to be extremely sensitive to inhibition by carbonyl reagents(7). The possibility therefore exists that the decrease in concentration of γABA results from lowered pyridoxal phosphate levels as a consequence of inhibition of pyridoxal kinase rather than from inhibition of the glutamic decarboxylase *per se.* This report demonstrates that pyridoxal kinase is much more sensitive than glutamic decarboxylase to inhibition by carbonyl reagents both *in vitro* and *in vivo.* These findings emphasize the possibility that inhibition of the kinase, with consequent decrease in pyridoxal phosphate production,

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* Supported in part by grant from Nat. Inst. Health, U. S. P. H. S.

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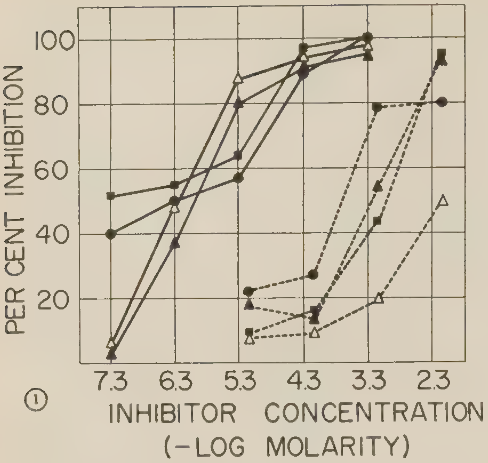
may be one mechanism by which certain carbonyl reagents limit production of γABA and produce *in vivo* other symptoms related to those of Vit. B_6 deficiency.

Methods. Adult rats received intraperitoneal injections of 1 ml of water or of aqueous solutions of test compound. In some instances urine was collected, chromatographed on paper, and chromatograms examined for pyridoxal catabolites by spraying with 2,6-dichloroquinonechlorimide(8). At specified time intervals, animals were decapitated and the desired organs removed, rinsed, blotted, weighed, and homogenized with 4 volumes of 0.05-0.1 M potassium phosphate, pH 6.5-7.0, or with 9 volumes of 0.25 M sucrose. Homogenates were centrifuged at 18,500 x g for 30 to 60 minutes to obtain supernatant solutions which were assayed both for pyridoxal kinase and for glutamic decarboxylase. Pyridoxal kinase in the phosphate buffer extract from beef brain was purified further by collecting that fraction of supernatant solution insoluble between 40 and 60% saturated $(NH_4)_2SO_4$ and dialyzing(7). Glutamic decarboxylase from beef brain appeared to be more stable in acetone powder extracts than in fresh tissue homogenates. Acetone powders were prepared in the usual way(9) and extracts were obtained by grinding the preparations with 0.1 M phosphate buffer, pH 6.4, containing 0.01% 2-mercaptoethanol and 5 mg of Tween 80 per ml. Protein was determined by the method of Lowry *et al.*(10). Incubation mixtures for pyridoxal kinase contained 5×10^{-4} M pyridoxal and ATP, 10^{-5} M Zn^{++}, 0.1 M potassium phosphate, pH 6.0-6.5, and enzyme with or without inhibitor. After incubating 1 hr at 37°C, the reaction was stopped by heating 3 min at 100°. When pyridoxine was used as substrate in place of pyridoxal, the product, pyridoxine phosphate, was converted to pyridoxal phosphate by a second incubation for 1 hr at 37° and pH 8 with an oxidase preparation from rabbit liver(11). Pyridoxal phosphate formation was followed by a modification of the apotryptophanase method of Wada *et al.*(12). Glutamic decarboxylase activities were measured by the fluorimetric method of Lowe *et al.*(13). A lower concentration of pyridoxal phosphate (0.02 mM in place of 0.5 mM) was used in incubation mixtures. This concentration was lowest that permitted optimal decarboxylase activity, and was used to insure maximum sensitivity of decarboxylase to inhibitors. The intensity of fluorescence of the product formed by reaction of γABA with ninhydrin was determined with the Aminco-Bowman spectrophotofluorimeter, set at activation wave length of 376 mμ and a fluorescence wave length of 452 mμ.

Results. Inhibitory concentrations of several carbonyl reagents for partially purified preparations of pyridoxal kinase and of glutamic decarboxylase from beef brain are compared in Fig. 1. The kinase is from 100 to 1000 times more sensitive than the decarboxylase to these inhibitory compounds.

When hydrazine was injected into rats, and activities of the 2 enzymes subsequently were compared, both enzymes were markedly depressed (Table I). Again the effect upon the kinase appears more severe. Under these same conditions, no significant inhibition of either kinase or decarboxylase was observed one hour after administration of isonicotinyl hydrazide (isoniazid), hydroxylamine, or pyridoxal oxime in amounts sufficient to give an initial "body concentration" in excess of 10^{-3} M. All of these compounds are potent inhibitors of the kinase *in vitro*(7), eliciting 50% inhibitions at concentrations between 5×10^{-7} and 5×10^{-6} M. The dihydrazone (azine) of pyridoxal, like hydrazine, is a potent inhibitor of pyridoxal kinase both *in vivo* and *in vitro* but does not inhibit glutamic decarboxylase under either condition. The time course of inhibition in liver and brain following injection of the azine is shown in Fig. 2.

Animals which received either hydrazine or hydroxylamine developed convulsions and died with larger doses. Hydroxylamine produced severe methemoglobinemia, and onset of convulsions was rapid. Neither pyridoxal azine nor pyridoxal oxime produced convulsions in amounts equimolar with convulsive doses of the free carbonyl reagents. Pyridoxal oxime was rapidly catabolized to 4-pyridoxic acid and its lactone, and these were



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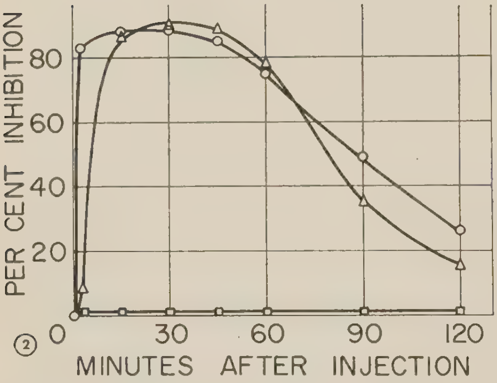

②

FIG. 1. Comparative effects of carbonyl reagents on activity of partially purified preparations of pyridoxal kinase and of glutamic decarboxylase from beef brain. Conditions are described under *Methods.* Solid lines show kinase activities, broken lines show decarboxylase activities. ●, Hydroxylamine; ▲, α-methylphenethylhydrazine; ■, semicarbazide; △, hydrazine.

FIG. 2. Effect of intraper. injections of pyridoxal azine on activities of pyridoxal kinase and of glutamic decarboxylase in livers and brains of rats as a function of time. Pyridoxal azine (15 mg) was administered as its hydrochloride by intraper. injections to 200-250 g female rats. Incubation conditions as described under *Methods.* Kinase activities in liver (○) and brain (△); decarboxylase activity in brain (□).

excreted in urine. Recovery of rats from an apparent weakness following injection of pyridoxal azine coincided with recovery of kinase activity.

Inhibition of pyridoxal kinase by pyridoxal azine *in vivo* could, in principle, result from its hydrolysis to pyridoxal and hydrazine with subsequent inhibition by the latter of the kinase during its assay *in vitro.* Such inhibition requires condensation of hydrazine with pyridoxal added as substrate, the resulting hydrazone or azine being the actual inhibitor (7). This possibility was eliminated by assaying the kinase with pyridoxine, which cannot condense with hydrazine, as substrate. The results (Table II) show that kinase levels are depressed about equally with either substrate; consequently inhibition by the injected pyridoxal azine cannot result from its preliminary hydrolysis to hydrazine.

Discussion. These findings demonstrate that inhibition of both pyridoxal kinase and glutamic decarboxylase can be produced *in vivo* by a single dose of some, but not all, of the carbonyl reagents that produce these effects *in vitro.* The mechanism of the 2 inhibitions is quite different. Carbonyl reagents inhibit pyridoxal kinase *in vitro,* and presumably also *in vivo* by combining with pyridoxal to form extremely inhibitory substrate analogues(7). That hydrazine inhibits *in vivo* thus infers that there are sufficient amounts of this agent and of free pyridoxal present in tissues to form the active inhibitor. Agents such as hydroxylamine and isoniazid, which inhibit the kinase *in vitro* but not *in vivo,* may be inactive under latter conditions because (a) they are not accumulated in tissues to the same extent as hydrazine, or (b) they require higher concentrations of pyridoxal or longer times to form the inhibitor, or (c) they are destroyed before they reach the site of inhibition. The latter explanation presumably holds for pyridoxal oxime, which rapidly gives rise to pyridoxic acid after injection. This immediate increase in excretion of pyridoxic acid was not observed following injection of pyridoxal azine, which inhibits *in vivo.*

Although these condensation products of carbonyl reagents with pyridoxal are potent inhibitors of the pyridoxal kinase, tests *in vitro* have shown them to be essentially without effect on glutamic decarboxylase. Carbonyl reagents inhibit this enzyme by condensing with pyridoxal phosphate to remove this functionally active coenzyme as a comparatively inactive coenzyme analogue. We prepared such analogues chemically and find them essentially without inhibitory activity

TABLE I. Effect of Hydrazine Injection on Levels of Pyridoxal Kinase and Glutamic Decarboxylase in Rat Tissues.

		Kinase activities*				Decarboxylase activities*	
		Liver		Brain		Brain	
Hydrazine inj., mg	Min. before sacrifice	Units/mg protein	Total units	Units/mg protein	Total units	Units/mg protein	Total units
0	0	11.6	7,140	23.4	834	216	7,380
		±3.5(σ)‡	±1,840	±5.2	±196	±37	±1,480
5	60	5.8	3,700	17.2	693	297	12,000
10	120	2.5	1,470	10.4	335	94	3,030
100	7†	.0	0	2.1	70	95	3,160

* One unit of activity is that amount of protein (in phosphate buffer extract) that forms one $m\mu$mole of pyridoxal phosphate or of γABA in 1 hr at 37° under assay conditions. Total activity is calculated on basis of total wet wt of organ.

† Time of death due to injected hydrazine.

‡ σ Stand. dev. with 10 control animals. Single animals were used for each level of hydrazine.

for either glutamic decarboxylase or for pyridoxal kinase. The fact that injected pyridoxal azine inhibits pyridoxal kinase, but not glutamic decarboxylase *in vivo*, is consistent with each of these observations.

Summary. 1. Carbonyl reagents such as hydrazine, hydroxylamine and isoniazid inhibit purified preparations of pyridoxal kinase and of glutamic decarboxylase from beef brain, but the kinase is 100 to 1000 times more sensitive. 2. Administration of hydrazine to rats decreases activity of these 2 enzymes in liver and brain; kinase levels are affected more severely. 3. In contrast to their effects *in vitro*, isoniazid, hydroxylamine, or pyridoxal oxime produced no significant inhibition of kinase or decarboxylase activity *in vivo*. Pyridoxal oxime appears rapidly catabolized *in vivo* since its injection results in extensive excretion of 4-pyridoxic acid and its lactone. Unlike free hydroxylamine, injection of pyridoxal oxime does not produce convulsions or extensive methemoglobin formation. 4. Similar administration of pyridoxal azine resulted in rapid and extensive decrease of pyridoxal kinase levels in liver, kidney, and brain. No effects on glutamic acid decarboxylase in brain were observed. 5. These observations demonstrate that individual carbonyl reagents inhibit quite different enzymes *in vivo*, and indicate that appropriate derivatives of such agents may show enhanced selectivity in their inhibitory actions.

TABLE II. Inhibition of Pyridoxal Kinase in Tissues from Rats Injected with Pyridoxal Azine.

	Inhibition of kinase, %* (30 min. after 10 mg dose)	
Organ	Pyridoxal as substrate	Pyridoxine as substrate
Liver	58	50
Kidney	63	62
Brain	6	3

* Avg values for 6 male rats (200-250 g each).

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Received May 13, 1960. P.S.E.B.M., 1960, v104.

Effect of Cortisone on Mice Infected with Chromogens and with Isoniazid Resistant *Tubercle bacilli.* (25906)

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D'Arcy Hart and Rees were the first to show that the severity of a tuberculous infection (H37Rv) in mice could be increased by concurrent administration of massive doses of cortisone(1). We were interested in investigating the effect of cortisone on experimental infections with chromogenic acid-fast and with isonicotinic acid hydrazide resistant (INHR) organisms.

Methods. Groups of 5 or 10 mice of the CF_1 strain, 16-18 g in weight, were infected by intravenous route with 0.25 ml of the appropriate culture. They were fed Purina chow *ad libitum* and weighed at weekly intervals. The strains of organisms used were: the human, virulent H37Rv; the H37Rv strain rendered resistant to 54 γ/ml of INH by serial passage in increasing quantities of drug, and 20 chromogenic acid-fast organisms of human origin obtained from Dr. Ernest Runyon. Cultures were grown in Dubos' albumin medium in Erlenmeyer flasks for 5 to 8 days depending on the strain, diluted with the same medium to give a reading of 65-70 in the Klett-Summerson colorimeter, then further diluted 1:4. Cortisone acetate suspended in 0.5% tragacanth, was administered by subcutaneous route for 7 days in a volume of 0.1 ml beginning the day of infection. Chromogen-infected animals that did not succumb were sacrificed between 6th and 8th week after injection. Mice infected with the INHR strain of H37Rv were observed for over 100 days and cultures made of spleens and lungs in Dubos' medium from sacrificed and from spontaneously dead mice. Survival time greater than or less than that of controls was not considered significant unless it exceeded 2 days. Autopsies were performed on some mice from all groups.

Results. As a base line for work with the other strains, we determined for an H37Rv infection minimal cortisone dose which when given for several days beginning the day of infection would result in significant decreased survival. Seven doses of 0.5 mg/day were required to produce this result (Table I). A dose of 0.25 mg for 7 days, though resulting in loss of weight, had no effect on survival, but did hasten death if given for 9 days. On the other hand, treatment with cortisone for a week *before* infection resulted in no increase in susceptibility. The 1 mg dose administered by gavage was probably insufficient to decrease survival markedly. Actual numbers of tubercle bacilli in mice treated from day of infection were found to be greatly increased even when the cortisone treatment was not lethal (Table II). Heavier growth occurred from spleens of treated than from those of infected, untreated mice sacrificed on 12th or 13th day after infection. This has been demonstrated more quantitatively by Batten and McCune(2).

Strains of tubercle bacilli highly resistant to INH produce chronic, slowly progressing disease in mice. Under our conditions, mortality was low and greatly delayed. Examples of the effect of cortisone on this infection are shown in Table III. Mortality is greater when the hormone is administered. That deaths were not due to other than acid-fast organisms was clearly shown in cultures of lungs

TABLE I. Effect of Varying Dosage of Cortisone on Infection with H37Rv.

Dose/day, mg	No. of doses†	Days' avg treated	Survival time, controls
.5	9	11.9	19.6
.25	9	12.6	21.0
.125	9	18.8	"
.025	9	21.0	"
.5	7	12.2	20.2
.25	7	18.6	"
1.0 *	9	17.6	19.6
.5	7‡	19.5	21.2
.2	7	22.0	"
.05	7	24.0	"

* By gavage.

† Administered by subcut. route beginning day of infection.

‡ Treatment started 7 days before infection.

TABLE II. Growth of H37Rv from Spleens of Cortisone-Treated and Control Mice.[*]

	Dilution of minced spleen									
	10^{-2}		10^{-3}		10^{-4}		10^{-5}		10^{-6}	
	Days of incubation									
	7	14	7	14	7	14	7	14	7	14
Cortisone, 7 doses, .5 mg	3[†]	>4	1	>4	0	>4	0	3	0	2
Control	2	4	0	3	0	1	0	0	0	0
Cortisone, 7 doses, .25 mg	3	>4	±	>4	0	4	0	2	0	1
Control	2	>4	0	3	0	1	0	0	0	0

[*] Dubos' albumin medium used. Growth recorded at 7 and 14 days.
[†] 0 = no growth; 4 = heavy growth, etc.

and spleens made to test the sensitivity of recovered tubercle bacilli. Contaminants were rarely present. Whether from sacrificed or spontaneously dead mice, these tissues yielded heavy cultures of acid-fast bacilli still completely resistant to 54 γ/ml of INH.

The atypical acid-fast organisms used in this study were not highly pathogenic for mice. A few deaths occurred in our animals infected with photochromogens No. 4 and 8 and not treated with cortisone (Table IV). The effect of cortisone administration is apparent. Virulence of strains No. 4 and 8 was enhanced as well as that of 4 additional cultures (No. 5, 6, 16, 26) as evidenced by increased mortality. If deaths occurred earlier than 16 days after infection, no lesions visible to the naked eye were observed in lungs. However, large numbers of acid-fast organisms were present in stained smears. When surviving mice were sacrificed 6 to 8 weeks after infection, lung lesions were found in those infected with cultures No. 1, 4, 8, 16, 18, 21 and 26, though not in every mouse of every group, whether they had received cortisone or not. In the case of culture No. 24, lung lesions were present only in cortisone treated animals. Kidney lesions were noted in mice infected with No. 4, 8 and 16, treated or not treated with cortisone. No. 21 and 26, on the other hand, did not provoke kidney lesions unless cortisone was administered. (More deaths might have resulted had heavier inocula been used. We preferred to use H37Rv as a reference point and to employ inocula of the same density as used in our routine tests with this strain.)

Discussion. The dose of cortisone used in these experiments to elicit an enhancing ef-

TABLE III. Effect of Cortisone on Infection with INH-Resistant H37Rv.[*]

Treatment	No. of doses	No. of mice dead	ST$_{50}$[†]	ST$_{100}$
Cortisone, .5 mg	7	7/10	47	>103
Controls	0	1/10	>108	
Cortisone, .5 mg	9	8/10	41	> 71
Controls	0	2/10	>110	

[*] Strain resistant to 54 μg/ml of INH.
[†] ST$_{50}$ and ST$_{100}$ = survival time in days of 50 and 100% of mice.

TABLE IV. Effect of Cortisone on Infections with Atypical Acid-Fasts.

Pkge. No.[*]	Group[*]	Deaths
1 & 1c[†]	photochromogen	0/5
4		2/10
4c		5/10
8		1/10
8c		9/10
16		0/10
16c		3/10
18 & 18c		0/5
21 & 21c		0/10
22 & 22c		0/5
24 & 24c		”
26		0/10
26c		2/10
5	scotochromogen	0/10
5c		3/10
6		0/10
6c		2/10
15 & 15c		0/5
19 & 19c		”
2 & 2c	Battey	”
3 & 3c		”
7 & 7c		0/10
17 & 17c		0/5
20 & 20c		”
23 & 23c		”
25 & 25c		”
9 & 9c	H37Rv	10/10
11 & 11c	*M. phlei*	0/10

[*] No. and group designations of Dr. Runyon's Sunmount collection.
[†] c denotes cortisone-treated mice, 0.5 mg/mouse /day for 7 doses.

fect on murine tuberculosis was greatly in excess of the equivalent dose customarily employed in management of human disease. On a weight basis, five-tenths mg/day of cortisone for a 20 g mouse for example, is 17 times the 100 mg/day maintenance dose frequently used for man.

The position of INHR organisms in human disease is still a matter of controversy although they have been isolated on primary culture from sputum and spinal fluid(3,4). The increase in virulence of INHR tubercle bacilli and of some chromogens when mice were treated with cortisone might have its parallel in human infections under conditions of prolonged stress or in anxiety states. In this connection, Steenken and co-workers have shown(5) that INHR organisms can cause progressive disease in silicotic guinea pigs.

Summary. 1) The virulence for mice of INHR laboratory strains of H37Rv was increased when cortisone was administered. 2) Cortisone also increased the pathogenicity for mice to some extent of 2 of 4 scotochromogens and 3 of 9 photochromogens tested. Seven Battey strains were unaffected.

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Received May 16, 1960. P.S.E.B.M., 1960, v104.

Stability of Isoniazid in Aqueous Solutions and Plasma. (25907)

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In our work on isoniazid (INH) blood level determinations in men and animals, the impression was gained that lower levels were found if the chemical test could not be completed immediately after blood sample was obtained. This raised the question of stability of the drug in solution. The following factors were considered: (a) Influence of temperature and pH in aqueous solution. (b) The possibility that the drug after becoming absorbed through the intestinal tract might be in a state in serum different from that in a simple solution; *e.g.*, INH could be bound by a protein fraction which might influence its stability.

Methods. The method used has been published(1). We have since modified this method as follows: Before medication was given patient's plasma was collected and used for blank and standard determinations to avoid the influence of the coloration by bile constituents which vary from one person to another.* Analytical error of the method is ± 0.3 μg. Two patients with polycythemia and healthy volunteers were available for blood donations. In each case 250 cc of blood was taken before breakfast and before medication for blank determinations. Ninety minutes after oral administration of 4 or 8 mg INH/kg another 250 cc of blood was obtained and first determination was made without delay. A pool of rabbit plasma was obtained from the carotid artery by exsanguinating male rabbits kept on a standard diet. Four rabbits received 20 mg INH/kg intravenously, their total blood was taken after 10 to 15 minutes and the plasma pooled. Another batch of pooled, untreated rabbit plasma was divided into 2 equal portions for the blank and for *in vitro* tests in which INH was added. Plasma specimens were kept at temperatures indicated in the tables, and INH determined at convenient intervals. Analog stability tests were performed in neutral aqueous solution and in buffered solutions at pH 4.5 to 8. While adequate quantities of blank and INH containing plasma were kept in bulk at temperatures as indicated, small samples of each sufficient for one test were kept frozen to avoid repeated melting and refreezing.

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* This same modification has been introduced, independently, by Dr. John W. Jenne, Veterans Adm. Hosp., Minneapolis, Minn. Personal communication.

Results. Aqueous INH solutions of 8 and 16 μg/cc kept at 37°, 25°, 7° and frozen showed no changes within 14 days. INH is stable in aqueous and buffered solutions at pH below 8 (Table I) and if stored at low temperature, hence standard solutions may be kept refrigerated for several weeks.

INH is not stable in plasma at any temperature. Rate of disappearance is greater at 37° than at low temperature (Tables II, III), but even in frozen state is significant (Tables IV, V). Plasma from rabbits or humans contains a substance which changes INH into a form not detected by the reaction used in the analytical method. It has been claimed that metabolites of INH are isonicotinic acid, acetyl INH and conjugates of isonicotinic acid (2,3). Neither of these gives this reaction. However, none of these metabolites have been identified satisfactorily.

TABLE I. INH in Phosphate Buffer Solutions. Added 8 and 16 μg/cc.

		μg found after days				
Temp.	pH	0	1	3	7	14
25°	4.5	7.9	8.0	7.8	8.0	7.9
	6	7.9	7.9	7.9	7.5	7.8
	7	7.7	7.9	7.8	7.7	7.6
	8	7.9	7.8	7.0	5.2	4.0
	4.5	16.0	15.8	15.5	15.5	15.8
	6	15.7	15.4	15.5	15.6	15.6
	7	15.8	16.0	15.8	15.4	15.5
	8	15.6	15.5	15.0	13.0	9.9
37°	4.5	7.9	8.0	7.8	7.9	8.0
	6	7.9	7.9	7.6	7.1	7.3
	7	7.7	7.8	7.5	6.8	6.5
	8	7.9	7.3	5.0	3.5	2.7
	4.5	16.0	15.8	15.3	15.5	15.5
	6	15.7	15.7	15.3	15.0	15.2
	7	15.8	15 7	15.3	14.9	14.2
	8	15.6	15.3	13.1	11.8	10.7

At temperatures of 7° or in frozen state the solutions showed no changes at pH ranges 4.5-8.

TABLE II. INH in Rabbit Plasma (3 Run Separately), Taken 30 Min. after Intravenous Injection of 20 mg/kg.

		Found μg INH/cc after days of storage			
Rabbit	Temp.	0	4	7	11
I	37°	22.4	20.3	5.4	1.0
II		20.4	18.6	10.2	9.6
I	7°	22.4	15.6	13.3	9.2
II		20.4	15.0	14.2	13.4
III		24.3	17.6	15.2	14.3

TABLE III. INH in Human Plasma, Taken 90 Min. after Oral Administration of 4 mg/kg (Patients) and 8 mg/kg to Volunteers.

			Found μg/cc after days of storage											
Patient	Origin of blood	Temp.	0	1	2	3	4	7	8	16	21	24	28	36
A	Polycythemia	25°		3.5					1.4	.8		0		
		7°		4.0					3.5	2.7		2.1	2.3	2.0
		frozen		3.7					4.0	3.4		3.1	2.9	2.6
B	"	25°	3.3	3.1	2.1		1.8			.02				
		37°	3.3	3.3	2.3		2.0			.4				
		7°	3.3	3.3	2.6		2.3			1.6				
		frozen	3.3	2.3	2.0		2.1			1.4				
Volunteer														
Normal		25°	6.1	4.7				3.9			3.0		2.1	
		37°	6.1	4.6				2.5			2.5		1.7	
		7°	6.1	4.7				3.2			2.7			
		frozen	6.1	4.7				3.4			3.0			
R		25°	5.4	4.9		2.8		2.6			1.0			
		37°	5.4	4.7		3.6		2.0			.0			
		7°	5.4	4.6		3.8		3.1			2.6			
		frozen	5.4	4.2		4.0		4.1			3.9			
J		25°	9.1	7.8		5.1		3.8			.0			
		37°	9.1	9.1		5.6		2.9			.1			
		7°	9.1	8.6		7.5		5.8			5.5			
		frozen	9.1	7.7		7.5		7.5			7.2			

This finding is important with reference to biological assay of INH in patients' plasma. For this assay the plasma usually is kept frozen or refrigerated for variable periods preceding testing. Adding the error caused by bacteriological technic of dilution it appears questionable whether the bioassay carries any value. Whether the factor contained in plasma is still active at the dilution resulting from distribution in the culture medium, cannot be answered because the chemical method is not sensitive enough for dilutions in that range. Should it be the case, INH would decrease in concentration during process of incubation, which usually lasts about 3 weeks. The presence of the postulated factor in plasma in larger or smaller quantities may explain why some patients show quicker inactivation of INH than others.

TABLE IV. 10 and 20 mg/cc INH Respectively Added to Pooled Rabbit Plasma.

	Found µg/cc after					
Temp.	4 hr	1 day	3 days	7 days	9 days	14 days
25°	7.2		4.4		2.2	2.0
37°	7.1		4.8		.3	.0
7°	7.9		5.9		3.2	3.0
	18.4	17.3	13.6	7.5		6.7
frozen	5.2		4.7		3.5	3.4

TABLE V. 10 mg/cc INH Added to Human Plasma.

		Found µg/cc after time				
	Temp.	0	1 day	3 days	7 days	21 days
R	25°	9.3	6.8	4.4	4.2	.9
R	37°	9.3	6.0	4.8	3.0	.0
J	7°	9.3	7.2	4.9	4.4	4.0
R	7°	8.8	7.2	6.1	5.8	5.1
R	frozen	9.3	8.0	6.9	6.0	5.7

Summary. INH is stable for several weeks in aqueous and buffered solution at pH values below 8 and at low temperature. It is unstable in human and rabbit plasma whether added to pure plasma or present after oral or parenteral administration. Instability is enhanced by increased temperature, but is quite marked at refrigerator temperature. The presence of a factor in blood causes changes in chemical state of INH so that it no longer reacts with reagent used in the assay method.

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Received May 20, 1960. P.S.E.B.M., 1960, v104.

Effect of β-3-Thienylalanine on Antibody Synthesis. I. Preliminary *in vitro* and *in vivo* Studies.[*] (25908)

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Experiments of Wissler *et al.* have shown that feeding β-3-thienylalanine (β-3-TA) in absence of dietary phenylalanine results in almost complete inhibition of antibody response to a single intravenous dose of particulate antigens in rats(1). In these experiments the amino acid analogue was incorporated in a liquid diet and fed by stomach tube for a period of 6 days beginning 2 days before antigen injection and continuing up to day before sacrifice. Antibody titer at this time was always considerably lower than that of control animals. The histologic transformations in rat spleen which accompany synthesis of antibody(2) were consistently absent in animals fed β-3-TA. Our experiments originated during study of antibody production in tissue culture(3). The purpose was to study the effect of β-3-TA on antibody synthesis *in vitro*

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[*] Supported in part by USPHS grant.

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TABLE I. Incorporation of Glycine 1-C^{14} into Antigen Bound Material. Ratio of radioactivity incorporation of experimental over control animals.

No. of spleens cultured	Days of β-3-TA feeding after antigen	β-3-TA added to culture	Exp. / Control
6	0,1,2	—	1.4
6	1,2	—	2.8
6	2	—	5.4
18		+	5.3
24		—	6.6

All rats inj. 3 days before sacrifice. All spleens cultured 3 days. β-3-TA added to tissue culture 12 hr after beginning of incubation. Amount of β-3-TA added was 50 γ/ml of medium (15 γ/mg of tissue present in culture).

and to compare this to inhibition of antibody synthesis observed *in vivo* by Wissler and coworkers(1).

Materials and methods. Young adult male albino rats of the Holtzman strain were used throughout. Tissue culture methods, manner of antibody titrations, of antibody determination in tissue culture fluids, and histological technics employed have been described(3-5). The antigen used in *in vivo* experiments was a 1% suspension of washed sheep erythrocytes; 1 ml of this suspension was administered intravenously. Tube-feeding of β-3-TA, diet preparation, etc. were done according to the methods described by Wissler *et al.*(1). Rats to be prepared for tissue culture were injected intravenously 72 hours before beginning of culture with 1.0 ml of *S. typhi* vaccine. Tissues to be cultured were always explanted 72 hours after antigen injection. β-3-TA added to the medium 12 hours after beginning of culture. The medium to which β-3-TA was to be added was prepared without phenylalanine.

Results. The initial experiments tested the effect of β-3-TA added directly to the tissue culture medium on synthesis of antibody as measured by incorporation of radioactive glycine into antigen-binding material. As shown in the 2 bottom lines of Table I, β-3-TA added to a tissue culture system in which spleen fragments were synthesizing antibody did not alter appreciably final antibody output.

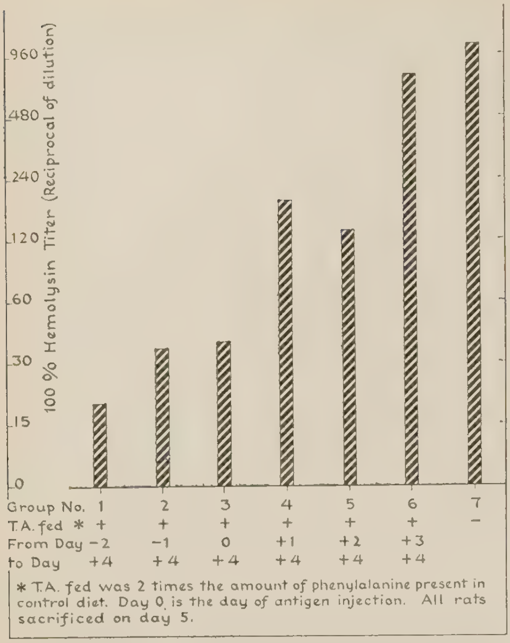
The fact that spleens for tissue culture were explanted 3 days after injection of antigen suggested the possibility that β-3-TA did exercise its depressing effect during earlier stages of antibody synthesis. Thus, β-3-TA would not depress the response to antigens once this response had been established. Experiments were therefore set up in which this hypothesis could be tested both *in vitro* and *in vivo*.

Table I summarizes results obtained in several tissue culture experiments, conducted as follows: β-3-TA was fed to rats for varying periods of time after antigen injection. Spleens were explanted 72 hours after antigen was administered and culture was allowed to proceed for 48-72 hours in normal medium. There is a marked depression of antibody synthesis if β-3-TA feeding is started on day of antigen injection or one day after (Table I). If feeding is begun on second day after antigen injection (1 day before starting tissue culture) or added to culture medium in which spleens from animals not receiving β-3-TA are explanted, there is only a very slight depression of antibody synthesis. In one experiment up to 7 times the amount of β-3-TA normally used was added to the culture medium and no more effect was noted.

To test further the possibility that β-3-TA was active only during early stages of antibody synthesis *in vivo* experiments were set up in which groups of rats were fed β-3-TA starting at different times in relation to antigen injection and continuing to day of sacrifice. Thus, it was hoped to be able to determine the period during which β-3-TA was most active in its depressing effect. Fig. 1 summarizes results obtained by this experimental approach.

If β-3-TA feeding is started after the end of induction period, there is no significant depressing effect on hemolysin synthesis. On the other hand, a considerable depression of the synthetic process is evident if β-3-TA is administered during the days immediately following antigen injection and an even greater depression exists with earlier feeding of the analogue.

To minimize the possibility of error arising from toxic phenomena due to β-3-TA all rats should receive the same amount of analogue. Thus rats in groups fed only for shorter times



Group No.	1	2	3	4	5	6	7
T.A. fed *	+	+	+	+	+	+	−
From Day	−2	−1	0	+1	+2	+3	
to Day	+4	+4	+4	+4	+4	+4	

* T.A. fed was 2 times the amount of phenylalanine present in control diet. Day 0 is the day of antigen injection. All rats sacrificed on day 5.

FIG. 1. Effect of β-3-TA feeding begun at different times before and after sheep erythrocyte inj. on 100% hemolysin titer in rats.

received a large dose of analogue over a shorter period of time. One may wonder whether under these conditions a stimulatory effect could be elicited on the synthesis of antibody. This seems very unlikely since no such effect was noted in tissue culture experiments in which β-3-TA was added to the *in vitro* system.

Discussion. Formation of antibody can be depressed or abolished by several physical(6) as well as chemical(7,8) agents. More recently reports have appeared which stress the fact that considerable inhibition or complete suppression of antibody synthesis can be obtained by use of substances which are analogues of normal metabolites(1,9-15). Only one of these papers(14) deals with the time at which the analogue seems more effectively to antagonize the synthetic process and bears out the same relationship between administration of analogue and inhibition of antibody production observed in the work reported here.

The so-called induction period of antibody formation is still a relatively poorly understood phenomenon. It is known that after a single antigenic stimulation, formation of antibody does not begin until approximately 48-72 hours after the stimulus has been introduced(16). During this time no appreciable antibody synthesis takes place as shown by studies of incorporation of labeled amino acids into antibody protein(17). The fact that β-3-TA seems to depress synthesis of antibody only when administered during induction period suggests that this substance may interfere with preparation of the so-called "antibody forming mechanism" or of a part of it. Sterzl's study in which 6-mercaptopurine(14), an analogue of natural purines, was used indicates that there may be two areas in preparation of this mechanism. In fact 6-mercaptopurine is known to interfere with synthesis of nucleic acids(18); β-3-TA seems to exercise its inhibitory function only at the level of protein synthesis(19). Further experiments are needed, and are being carried out at present, to clarify the level in the antibody synthesizing process at which β-3-TA acts. These studies may contribute greatly to a better understanding of the early stages of antibody synthesis.

Use of β-3-TA in experiments of this nature may be made somewhat cumbersome by the need to administer the analogue by stomach tube. This disadvantage may be compensated by the fact that β-3-TA affords a tool which has no equal because of its lack of toxicity for normal cells at the level used in these experiments(1). The possibility of β-3-TA administration by a route other than oral in the hope of further simplifying its use is being investigated.

Summary. When β-3-thienylalanine, an analogue of phenylalanine, is fed to rats for appropriate time before and after antigen injection it will markedly inhibit antibody response. This analogue added to an antibody synthesizing tissue culture system gave no inhibition of antibody synthesis under these conditions. It appears that unless β-3-TA is fed during induction period, its inhibitory effect will not be manifested. It is possible then that β-3-TA is active only in inhibiting the "setting up" of the antibody forming mechanism.

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Received May 20, 1960. P.S.E.B.M., 1960, v104.

Immunization Studies with Living Vaccine of *Salmonella typhimurium.* (25909)

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Production of artificial immunity to Salmonella infections in both man and animals is a problem which for the most part remains unresolved. Only recently has a properly controlled field study shown that typhoid vaccination confers some level of protection against infection in man(1). In laboratory animals, some information indicates that recovery from the disease yields best immunity to reinfection. In the past, such results were explained on basis of selection of innately resistant animals initially present(2). However, Hobson(3) recently showed that mice infected with relatively avirulent strain of *Salmonella typhimurium* developed a resistance to reinfection with virulent strains without appreciable deaths occurring from initial infection. Studies using killed *S. typhimurium* vaccines to immunize against mouse typhoid demonstrated that these vaccines offer only low level of protection against infection under natural conditions(4) and are only somewhat more satisfactory against challenge by intraperitoneal route(5). In fowl typhoid, where recent studies have indicated that naturally recovered chickens are immune to reinfection with *Salmonella gallinarum*(6), Smith(7) showed that dead vaccines had little or no protective effect while good immunity was produced by live vaccines. In view of these findings, and since our previous attempts to protect against mouse typhoid with killed vaccines have been unsuccessful, a living avirulent mutant of *S. typhimurium* was sought for further investigation. Isolation of streptomycin-dependent bacterial strains by Miller and Bohnhoff(8) suggested a technic which could be employed to obtain an avirulent mutant of *S. typhimurium*. Herzberg and Elberg(9,10) reported isolation and characteristics of such mutant of *Brucella melitensis* and on use of this variant in immunization studies. This communication gives results in which living vaccines were employed to protect mice against intraperitoneal challenge with a virulent culture of *S. typhimurium*.

Materials and methods. Cultures. An antigenically complete culture of *S. typhimu-*

rium (1,4,5,12;i,1,2) designated as strain 10, was obtained from culture collection of Walter Reed Army Inst. of Research. Within 7 to 14 days intraperitoneal injection of as few as 10^2 cells of this strain resulted in death of 90-100% of mice of the Bagg strain. This culture and a streptomycin-dependent (S^d) mutant derived from it were employed in initial experiments. The S^d mutant was obtained by plating approximately 5 x 10^{10} cells of strain 10 on nutrient agar containing 0.6 mg/ml of streptomycin sulfate (Pfizer). A colony was observed after 5 days incubation at 37°C which, on further testing, proved a streptomycin-dependent, but otherwise unchanged, derivative of parent culture. The cultures were grown on nutrient agar (Difco), or with S^d variant of strain 10, on nutrient agar supplemented with 0.6 mg of streptomycin/ml. *Immunization tests.* Living vaccines were prepared from washed cells of 16 hour cultures standardized turbidimetrically to contain 10^9 bacteria/ml. Killed vaccines were obtained by heating aliquots of living preparation at 56°C for 1 hour. Mice were immunized with 0.5 ml of appropriate vaccine intraperitoneally and challenged by same route with 5 x 10^7 cells of *S. typhimurium* strain 10 eighteen days later. This high challenge dose was chosen to provide a stringent test for the vaccine thereby eliminating evidence of low level immunity previously reported(5).

Results. Preliminary experiments demonstrated that some mice injected intraperitoneally with 10^7 washed S^d cells of *S. typhimurium* died in 3 weeks following inoculation. Post-mortem cultures revealed presence of streptomycin independent *S. typhimurium*. *In vitro* tests were then carried out to determine frequency of reversion of the S^d strain to streptomycin independence. When 10^8 washed S^d cells were plated on nutrient agar lacking streptomycin, approximately 4-5 colonies appeared in 24 hours. These colonies were independent of and sensitive to streptomycin and were in sharp contrast to the slow residual growth of the cell population plated, occurring probably due to presence of intracellular streptomycin not removed by the washing process. As a result of this residual growth, further reversions to streptomycin independence were observed during the next few days of incubation. In view of the high virulence ($LD_{90-100} = 10^2$ or fewer cells injected intraperitoneally) of the strain of *S. typhimurium* employed, the presence of even one reverted cell is undesirable.

It is known that purine-requiring mutants of *S. typhosa* and *Klebsiella pneumoniae* do not multiply in the peritoneum of mice due to absence of purines in this region(11,12,13). Thus, to minimize or effectively eliminate reversions from avirulent to virulent state *in vivo*, a streptomycin-dependent, purine-requiring mutant of *S. typhimurium* was isolated by subjecting the S^d strain to ultraviolet irradiation followed by penicillin technic of Lederberg and Zinder(14). Studies to determine frequency of back mutation of this 'double mutant' to the purine-streptomycin independent state indicated that such double reversions could not be detected by either *in vitro* or *in vivo* procedures.

A bacteriologic study of the fate of this double mutant strain following intraperitoneal injection into mice was then undertaken. The number of organisms of this strain which could be administered without causing deaths in mice was of the order of 5 x 10^8 bacteria. Blood, liver, spleen and peritoneal contents of groups of 10 mice were examined by streaking from these areas on plates of nutrient agar containing streptomycin at daily intervals following inoculation of the cells. The results indicated a clearance of organisms within 72 hours, such that no instances of positive blood or spleen cultures were detected with the method after 72 hours. At this time the peritoneal fluid was also free of organisms. When bacterial counts were made on individual whole mice at periods after immunization using Waring blender technic(15), a decrease in total number of organisms from original inoculum of 5 x 10^8 cells to 1 x 10^5 cells occurred at the end of 7 days with a further decrease to about 5 x 10^1 cells at the end of 18 days. This double mutant strain was then employed in immunization experiments.

Groups of mice were immunized with a single injection of either the double mutant, a

TABLE I. Comparison of Protection Afforded Mice against Intraperitoneal Challenge by Heat-Killed and Living Vaccines of *S. typhimurium*.

Vaccine	Deaths/Total*
Heat-killed *S. typhimurium*, wild type	22/48
Heat-killed *S. typhimurium*, double mutant (S^d Pur^-)	20/49
Live *S. typhimurium*, double mutant (S^d Pur^-)	20/50
Heat-killed *S. flexneri*	47/47
No vaccine	49/49

* Challenge dose = 5×10^7 *S. typhimurium* administered intraper. 18 days after immunization with 5×10^8 cells of the vaccines.

heat-killed preparation of these same cells, or a heat-killed *Shigella flexneri* vaccine. The *S. flexneri* vaccine was included as a control against possible development of a non-specific immunity similar to that described by Landy and Pillemer(16). Immunized mice and untreated controls were challenged 18 days after immunization with 5 x 10^7 cells of the virulent, wild type strain of *S. typhimurium*. The results showed that when this high challenge dose was employed, the living vaccine was not superior to heat-killed vaccine in protecting mice. A typical protocol is presented in Table I.

Discussion. These studies confirm observations that heat-killed vaccines of *S. typhimurium* do not confer a high level of protection on mice against intraperitoneal challenge. An attempt was made to determine if living vaccine would yield better results. However, when a streptomycin-dependent mutant of a highly virulent strain was used as living vaccine, it proved unsafe; the combination of a relatively high reversion rate to streptomycin independence and high virulence of the streptomycin independent form was sufficient fatally to infect an appreciable number of mice receiving this preparation. To prevent reversion to the virulent state, addition of a purine requirement to the streptomycin-dependent strain was accomplished yielding a culture which was safe for use in mice as a living vaccine. Nevertheless, this immunizing agent did not prove to be significantly better than heat-killed vaccine in protecting animals against challenge doses administered by the intraperitoneal route.

Summary. A streptomycin-dependent mutant was isolated from a virulent strain of *S. typhimurium* for evaluation as a living vaccine in mice. *In vivo* and *in vitro* studies indicated that this strain was unsafe as an immunizing agent due to reversions to streptomycin independence. A mutant of the streptomycin-dependent strain was obtained requiring purine, a nutritional factor absent in mouse peritoneum. Reversions of this double mutant strain to the purine-streptomycin-independent state could not be detected. The results of experiments comparing this living vaccine with heat-killed preparation indicated that the living vaccine was not superior in protecting mice.

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Received May 23, 1960. P.S.E.B.M., 1960, v104.

Enhanced 5-Nucleotidase Activity Associated with White Muscle Disease.* (25910)

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Histochemically it has been shown that the proliferation of connective tissue characteristic of human muscular dystrophy is accompanied by an appreciably greater 5-nucleotidase reaction than is present normally(1). This observation prompted an investigation of the status of this enzymatic aberration in tissues from lambs previously characterized relative to affectation by white muscle disease (WMD)(2). Results of such studies are reported here.

Materials and Methods. The assay procedure used includes experimentally indicated departures from conditions specified for other tissues(3). Three g samples of minced semitendinosus muscle were taken in duplicate from polyethylene packaged tissues which had been stored frozen up to 18 months beyond necropsy. The tissue was comminuted in 0.05 M NaCl-0.005 M Tris buffer, pH 8.3 using a glass mortar and pestle and a Potter-Elvehjem homogenizer. The supernatant fraction from 15 minutes centrifugation at 5,000 × g was diluted with buffer to 10 times the weight of the tissue. One-half of this extract was dialyzed 20 hours against 25 volumes of buffer which was changed once. After recentrifugation, the supernatant fraction of the dialyzate was diluted with buffer to 20 volumes relative to the original sample weight. All steps were performed at 0-5°C. Aliquots representing 100 mg tissue equivalent of the freshly prepared or dialyzed extracts were assayed separately. The nitrogen contents of each enzyme preparation and of the residue from initial centrifugation were determined by the Kjeldahl procedure. Three incubation mixtures were used in assaying the hydrolytic activity in each enzyme preparation, one containing no substrate, one containing adenosine-5′-monophosphate (AMP) and one with p-nitrophenyl phosphate (NPP). Incubation was carried out in 45 ml glass-stoppered centrifuge tubes containing 0.051 M Tris buffer, pH 8.3, 0.01 M NaCl, 29 μmoles substrate (if any) and enzyme in a total volume of 10 ml. Two ml aliquots of brei were withdrawn and added to 8 ml 10% trichloroacetic acid (TCA) immediately after adding enzyme and again after a one hour incubation at 37°C with shaking. Phosphorus contents of TCA filtrates were determined by the Fiske-Subbarow procedure. Net amount of phosphorus liberated from each substrate was taken as the final less the initial values, further corrected for amount released in absence of substrate. The latter values were consistently negligible.

Results. Under the conditions described, hydrolysis of AMP was linear during the incubation period and was proportional to enzyme concentration. The enzyme was saturated at concentration of AMP used. A rather broad pH optimum was found for AMP hydrolysis between 8.8 and 7.8, but the activity decreased markedly towards pH 7.5. Activity against NPP, however, was minimal at pH 8.3. Dialysis resulted in virtual elimination of NPP hydrolysis while consistently enhancing that of AMP.

On a tissue equivalent basis, the full activity of homogenates was retained in supernatant fractions up to 25,000 × g. Prior autolysis of several homogenates for 3 days at 70°F under chloroform(3) resulted in substantially reduced activity. Rehomogenization of initial tissue residues did not materially change the ratios of activities obtained with diseased and normal tissues.

The 5-nucleotidase activities found in tissues from normal and WMD lambs necropsied from 2 to 6 weeks of age, and in tissues from their twins sacrificed at less than one week of age(2), are shown in Table I.

Compared to the notably constant 5-nucleotidase activity in tissues from control lambs,

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* Technical Paper No. 1304, Oregon Agric. Exp. Station.

TABLE I. 5-Nucleotidase Activity of Semitendinosus Muscle of Normal and White Muscle Diseased Lambs.

Calculation basis	Enzyme preparation	Substrate	μmoles P released/hr* — Age — Lot 1—Normal control† 2-6 wk [10]	Lot 1—Normal control† <1 wk [8]	Lot 2—WMD basal 2-6 wk [9]	Lot 2—WMD basal <1 wk [6]
100 mg tissue	Dialyzed	AMP	1.2 ±.06 (.9-1.5)	1.6 ±.2 (.9-2.3)	6.4 ± 1.4‡ (2.0-14.3)	1.7 ±.2 (1.0-2.1)
Idem	Fresh	"	1.0 ±.1 (.4-1.4)	1.5 ±.2 (.9-1.9)	5.8 ± 1.3‡ (1.0-13.3)	1.5 ±.1 (.9-1.7)
"	" §	NPP	.8 ±.06 (.5-1.1)	.6 ±.1 (.4-1.1)	.4 ± .1 (.1- .9)	.7 ±.2 (.1-1.7)
mg of extract N	Dialyzed	AMP	2.6 ±.2 (1.9-3.6)	3.9 ±.5 (2.2-5.7)	14.8 ± 3.4‡ (3.8-32.2)	4.8 ±.5 (2.9-6.1)

* Mean ± stand. error. Ranges among lambs in parentheses.
† Lots 1 and 2 as reported previously(2). No. of lambs in brackets.
‡ Significantly greater than Lot 1, 2-6 wk ($P < .01$).
§ Hydrolysis of NPP by dialyzed preparations uniformly <.1 μmole P/hr.

WMD tissues exhibited a range in values up to 12-fold the normal average, probably related to stage of the pathological process(4). The differences were no less when expressed on an enzyme nitrogen basis, or when related to the dry matter or nitrogen contents of the tissues. There was little difference in activities in tissues from the twins of normal and WMD animals, sacrificed at a few days of age.

Nonspecific phosphatase activity against AMP, as measured by NPP hydrolysis(3), could be virtually restored in dialyzed enzyme preparations by addition of 1 x 10^{-3} M Mg^{++}. An apparent Mg^{++} stimulation of 5-nucleotidase activity in nondialyzed preparations could be accounted for by increases in NPP hydrolysis, as could additional stimulation in combination with 1 x 10^{-4} M ethylenediamine tetracetic acid. The latter alone, however, inhibited both hydrolyses severely. Calcium ions added up to 0.1 M were without effect on enzyme preparations from either normal or WMD tissues.

These results lend credence to the earlier postulate of the possible importance of the 5-nucleotidase reaction in soft tissue calcification(5). Considering WMD in part as a growth aberration(6), the reaction could conceivably assume a role in deranged nucleoprotein and protein syntheses. Levels of 5-nucleotidase activity in tissues from lambs variously responding to selenium and vitamin E therapy(2,6), are currently under study. Similar studies are also being made of analogous conditions with respect to rat liver necrosis(7).

Summary. Suitable conditions for assay for 5-nucleotidase activity in lamb skeletal muscle tissue are described. The activity of this enzyme was significantly greater in tissues from lambs affected by white muscle disease than in tissues from normal lambs.

The authors acknowledge efficient technical assistance of Pauline Fleetwood.

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Received May 23, 1960. P.S.E.B.M., 1960, v104.

Rash Induction by Intracellular Products of Group A Streptococci.* (25911)

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Extensive work(3,4,5,12) has been done on electrophoretic separation of intracellular components of group A streptococci. However, there are fewer reports on biologic activity *in vivo* of such fractions(11). This study was undertaken to investigate chemical separation, antigenic constitution and intradermal biologic activity of fractions obtained by starch electrophoresis.

Materials and methods. Preparation of streptococcal extracts. Five to 6-hour cultures of group A streptococci, types 18 and 4 and strain NY5 were grown in a completely dialyzable medium(13). After harvesting the bacteria by centrifugation in a Sharples, they were washed 3 times with distilled water and resuspended in distilled water, 30 g wet weight plus 100 ml water. Thirty-ml aliquots were disrupted in a 9 kc Raytheon, 2½ to 3 hours, 130 plate voltage, temperature 4°C. Sonic extracts were cleared of cellular debris by centrifugation 1 hour, 12,800 to 15,000 g in a refrigerated Serval. Supernates were dialyzed against saline or balanced salt solution for 24 hours at 2°C, filtered through an 02 Selas filter, placed in ampules and frozen at –70°C. Similar sonic extracts of *Salmonella typhosa* Ty2 were prepared. Bacteria grown on veal infusion agar were exposed to sonic oscillation for ½ hour. *Fractionation of sonic extracts.* One streptococcal type, 18, was separated by starch electrophoresis(7), veronal buffer pH 8.6, ionic strength 0.1, potential gradient 500 volts, 16 hours. After completion of run, the block was cut in 1½ cm strips, from point of origin. Five to 6 blocks were done in succession, comparable strips pooled and eluted with 100 ml distilled water by washing on a sintered glass filter of medium porosity under suction. Pooled fractions were concentrated to about ⅓ the original volume of extract by ultrafiltration(2). Starch control fractions were processed in the same way. All procedures were done in a 1°C cold room. An aliquot of each fraction was saved for chemical analyses; the remainder was dialyzed against 0.85% saline, placed in ampules and frozen at –70°C. *Chemical analyses of fractions.* Each pooled fraction was analyzed for RNA by the orcinol method (1), for total phosphorus by the method of Fiske and Subbarow(6), for DNA by the diphenylamine reaction(1) and tyrosine by the Folin-Ciocalteau method. *Serologic activity of fractions.* Each fraction was checked with T18 type specific antiserum, group A specific antiserum, and with antiserum prepared against either viable T18 streptococci or against T18 streptococcal sonic extracts. The Ouchterlony technic was used. *Biologic activity of sonic extracts and fractions obtained by starch electrophoresis.* Injections of whole sonic extracts, 1.0 to 1.5 ml, and fractions 0.5 ml, were made intradermally on the shaved abdominal surface of American Dutch rabbits, average weight 1 kg. *Intradermal control injections.* Intradermal injections of 1.0 ml were made into rabbits with sterile balanced salt solution; sterile 0.85% saline; trypsin (Difco) 5 mg/ml in balanced salt solution, final pH 7.8; histamine acid phosphate 0.275 mg/ml suspended in 0.9% saline equivalent to 0.1 mg histamine/ml (Eli Lilly and Co.); concentrated extracellular products of T18 containing 4 mg lyophilized material per ml; supernate from rabbit epithelial homogenate with and without added cysteine.

Results. Chemical analyses of fractions. These data are given in Table I. *Serologic study on fractions.* Fractions 1 through 7 taken from the positive side of the origin formed multiple lines of precipitate with T18 antiserum (Fig. 1). No lines of precipitate formed with fractions beyond 8 or with fractions taken from the negative side of the origin. Fractions 0 through 7 reacted with type specific M antiserum, fractions 0, 4, 5, 6, 7 forming one line of precipitate, fractions 1 and 3 two lines, and fraction 2 three lines.

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* Supported by a grant from USPHS.

TABLE I. Chemical Analyses on Fractions from Starch Electrophoresis.*

	μg/ml			
No. fraction†	Tyr	P	DNA	RNA
−1 through −5	—	—	—	—
0	—	—	—	—
1	28	—	—	—
2	100	9	—	—
3	310	43	—	92
4	460	127	—	800
5	400	290	—	3160
6	240	404	—	5000
7	53	210	85	2580
8	—	91	440	660
9	—	54	610	190
10	—	28	360	12
11	—	8	120	—
12 through 16	—	—	—	—

* Tests on starch control fractions read between .0-.4 O.D. In reading test fractions, starch control fraction was set at .0 O.D.

† Fractions taken from negative side of origin (0) are designated as minus.

Only fractions 1 and 2 reacted with group A specific antiserum. One broad line of precipitate was formed with each of the 2 fractions.

Intradermal activity of sonic extracts and fractions. Slight to considerable edema developed 3 to 6 hours after injection of sonic extracts and certain fractions into rabbits. Fractions 0 through 8 were the most active in eliciting an early reaction. The edema gradually subsided during the next 24 to 48 hours. Approximately 96 hours post-injection, 2 types of lesions began to develop in some of the animals, a remittent nodular reaction, grossly resembling that previously described by Schwab and Cromartie(9,10) and a spreading erythematous eruption resembling that described years ago by Rivers(8). The latter lesion appeared the 3rd to 4th day post-injection. Its advancing border was marginated, slightly raised from the normal surrounding skin. There was very little edema or induration. The bright red lesion faded the 6th to 8th day. Heavy desquamation of the involved area began the 9th to 11th day. After the original lesion faded, a fresh, bright red eruption (secondary lesion) occurred adjacent to the original lesion (primary lesion) in about 10% of the reactants. Several days later the new extension faded and eventually desquamated. Rarely a tertiary extension adjacent to the secondary lesion occurred about the 10th to 11th day post-injection. Tables II, III, and IV present these data. Figs. 2 and 3 show the reaction.

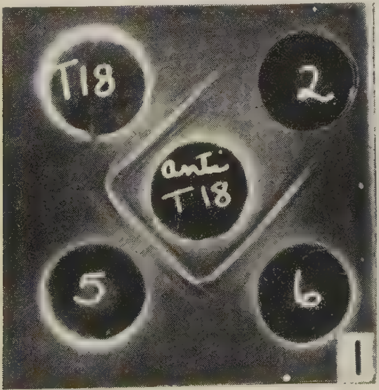


FIG. 1. Ouchterlony plate showing antigenic heterogeneity of starch fractions. Center well antiserum to T18 extract. Peripheral wells, crude sonic extract of T18 and fractions, as numbered.

TABLE II. Intradermal Activity of Strep. Extracts in Causing an Erythematous Eruption.

Streptococci	No. of rabbits	No. developing lesion		
		Primary	Secondary	Tertiary
T4	10	5	—	—
NY5	47	25	—	—
T18	142	62	10	4

Studies on erythematous eruption. Upon centrifugation of crude sonic extracts at 105,400 g for 1 hour in a refrigerated Spinco, both sediment and supernate were active in producing the eruption; the activity causing recurrent nodular formation was concentrated primarily in the sediment. By intradermal injection of supernate only, the erythematous

TABLE III. Erythematous Eruption Caused by I.D. Injection of Certain Fractions.*

	No. of rabbits developing lesion	
No. fraction	Primary lesion	Secondary lesion
C†	—	—
1	5	—
2	5	—
3	5	2
7	5	—
8	2	—
10	2	—
13	2	—

* Result of 2 separate experiments. Fractions −4 through 15 were each inj. into 9 rabbits. All fractions not listed did not produce this reaction.

† Starch control.

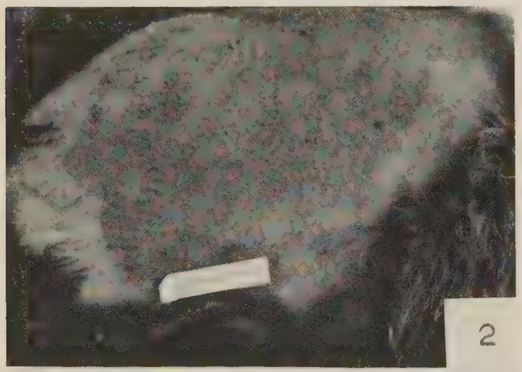
TABLE IV. Nodule Formation Caused by I.D. Injection of Certain Fractions.*

No. fraction	No. of rabbits developing nodules
C†	—
1	4
2	8
3	8
4	3

* As under Table III. † Starch control.

eruption occurred in absence of nodular formation.

To determine extent and duration of capillary damage, after development of the eruption, 5 ml of a 1% saline solution of trypan blue was injected intravenously. The first 2 days after appearance of the lesion, the dye appeared immediately over the eruption area. On the 4th-8th day, accelerated appearance of dye occurred only at the periphery of the lesion. By the 9th day there was no accelerated appearance of dye, the animal became uniformly blue as time elapsed.



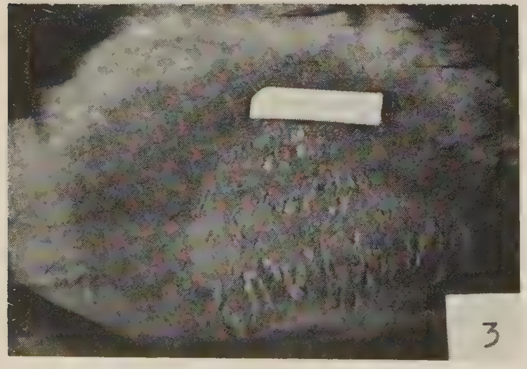


FIG. 2 (top). Primary erythematous eruption showing marginated, slightly raised advancing border.

FIG. 3 (bottom). Fresh secondary extension of the eruption adjacent to the now desquamating original lesion, 6 to 8th day post-inj.

The variability in rabbit response made it difficult to study the mechanism of the reaction. Using the same extract, rabbits showing a positive reaction varied from 8 out of 10 to 0 out of 10. The variability was not correlated with aging of the extract. The lesion could be the result of a humoral or delayed type hypersensitivity present in only certain rabbits. Therefore 2 groups of rabbits were given multiple subcutaneous and intradermal injections over the back and legs with equal parts of Freund's complete adjuvant and streptococcal extract. Three weeks later both groups reacted with strong Arthus reactions to an intradermal injection of homologous sonic extract. One group, 6 out of 11 rabbits, developed a rash with 2 of the reactants showing a secondary extension of the lesion. In the other group, 0 out of 8 rabbits developed a rash. The presence of circulating antibody did not insure either production of a positive reaction or inhibition of the reaction.

Intradermal activity of control injections. These data are given in Table V. Only *S. typhosa* extract produced a lesion similar to streptococcal extracts.

TABLE V. Intradermal Activity of Control Injections in Causing an Erythematous Eruption.

I.D. injection	No. of rabbits	No. with typical reactions	No. with minimal reactions*
Conc. extracellular products of T18	10	0	0
Histamine	"	0	2
Epithelial homogenate	"	0	1
Idem with cysteine	"	0	3
Trypsin	" †	0	1
Saline	29	0	7
Balanced salt solution	53	0	6
S. typhosa sonic extract	10	3‡	

* Streaks of erythema; similar reactions in test animals were called negative.

† Within 5 to 15 min. after inj., all rabbits developed hemorrhage at inj. site. This reaction proceeded to lysis of epithelium during next hours.

‡ Two of the 3 reactants developed a secondary extension of the reaction. All developed a necrotic lesion. None formed remittent nodules.

Discussion. By sonic disruption of bacteria, a heterogenous population of macromolecules is probably obtained. Thus, common antigenic and toxic groups might be attached to molecules of various sizes and charges which would be dispersed widely in starch electrophoresis. The antigenic heterogeneity of the fractions and wide dispersal of the active material causing the erythematous eruption substantiate this idea.

Rashes and eruptions are found in many human streptococcal infections. This reaction resembles the eruption seen in human erysipelas. The eruption may be due to a preexisting state of hypersensitivity in some of the rabbits or to induction of a hypersensitive state by the streptococcal injection. Alternately the streptococcal injection may activate release of deleterious host products which in turn produce the reaction. Intravenous injection of trypan blue revealed persisting capillary damage at periphery of the lesion at a time when the secondary eruption normally occurred. The latent period necessary for production of the rash supports either theory. The negative results obtained in this study following both lines of reasoning do not necessarily rule out either hypothesis.

Summary. Intradermal injection of crude sonic extracts and fractions obtained by starch electrophoresis of group A streptococci produced a spreading erythema in a variable percentage of rabbits. Recurrence of the lesion developed in about 10% of rabbits after abatement of primary lesion. The variability in rabbit response made it difficult to characterize the active principle and to determine the underlying mechanism of the reaction.

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Received May 23, 1960. P.S.E.B.M., 1960, v104.

Production of Normal-Lived Erythrocytes with Erythropoietin.* (25912)

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Although erythropoietic hormone may be an important factor in normal regulation of erythropoiesis, it can be obtained in significant amount only from animals whose erythropoiesis is markedly disturbed(1). If erythropoietin is part of the normal mechanism of erythropoiesis, one would expect administration of this hormone to result in production of normal erythrocytes. If production of erythropoietin is an emergency measure designed to flood the circulation with hastily or incompletely formed red cells, one might expect to find evidence of this in abnormal morphology, incomplete or abnormal hemoglobinization, increased fragility, or in shortened average survival time in the circulation. Gordon *et al.*(2) investigated the properties of erythrocytes produced under the stimulus

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* This work supported in part by funds from U. S. Atomic Energy Comm.

of human urinary erythropoietin and found normal osmotic fragility and oxygen carrying capacity and no increase in amount of abnormal hemoglobin. However, because it has been suggested(3) that erythrocytes with an abnormally short life span are produced after acute bleeding (a time when erythropoietin is found in high concentration in the circulation), it was considered important to determine the *in vivo* survival of erythrocytes produced under the influence of administered erythropoietin.

Material and methods. Erythropoietin used was obtained by the same method and from the same patient described earlier(4). By direct comparison of methods, it has been determined that 1 mg of human urinary erythropoietin used for the published dose response curves(4) is equivalent to approximately 7 cobalt units as described by White *et al.*(5). Two groups of 10 normal female rats of Long-Evans strain with an average weight of 187 g were used in this study. One group was given the equivalent of 6 cobalt units of human urinary erythropoietin subcutaneously daily for 10 days. On the 5th day (in middle of injection period) both groups were given 10 microcuries of glycine-2-C^{14} intraperitoneally. All animals were weighed periodically so that an accurate correction could be made for dilution of specific activity due to blood volume increase. In order to minimize blood loss due to sampling, only 5 animals of each group were sampled at each interval; 0.25 ml samples were drawn from the tail vein into a heparinized syringe. The cells were washed 3 times with physiological saline and specific activity of $BaC^{14}O_2$ prepared from hemoglobin determined as described previously (6).

Results. A single microhematocrit determination was done the day after last injection of erythropoietin (on the 11th day). The average hematocrit for the injected group was 57.3 ± 2.1 as compared to 44.3 ± 0.46 for controls, indicating that erythropoiesis had been effectively stimulated. After 14 days of treatment with the same dose of erythropoietin one would expect approximately a 40% increase in total circulating red cell volume (4). A growth curve was kept during the course of the experiment and the value obtained for each sample was corrected for dilution due to growth. A further small correction was made for loss of activity due to sampling. The results are presented in Fig. 1. The injected group incorporated more than twice as much glycine into red cells as did the controls, substantiating the fact that erythropoiesis had been accelerated at time of glycine administration.

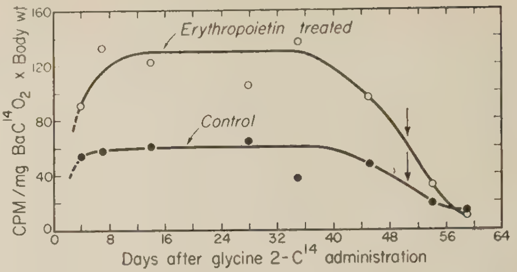

FIG. 1. The shape of both curves is compatible with finite life span and the point at which specific activity falls to ½ the plateau value is the same (51 days) for both groups.

The shape of both curves is compatible with a finite life span and the point of inflection or point at which specific activity falls to ½ is the same for both groups. The time at which level of activity fell to ½ the plateau value was 51 days in both groups. This value is in fair agreement with previously published data from this laboratory(7,8,9) using glycine-2-C^{14}, but is less than the recent 59 day estimate of Belcher and Harriss using Fe^{59} and a Bartonella-free strain of rats(10).

Discussion. Erythropoietic hormone assumes a greater physiological importance if it can be established that it represents part of the normal mechanism by which red cell production is controlled. Cells formed under influence of this hormone are indistinguishable from normal erythrocytes as far as survival time in circulation, pattern of hemoglobin electrophoresis(2), osmotic fragility and oxygen carrying capacity(2). Alpen *et al.*(11, 12) emphasized that both bleeding and human urinary erythropoietin administration cause a greatly increased output of erythrocytes, not by any change in marrow dynamics, but by stimulating stem cell division. An effect at the stem cell level would be expected to result in production of normal erythrocytes. Thus, there is no evidence to indicate that erythropoietin is part of an abnormal mechanism.

Summary. Erythrocytes produced in rats under stimulus of human urinary erythropoietin have a normal survival time in circulation. This and other evidence suggest that erythropoietin administration results in production of normal erythrocytes. This evidence is compatible with the concept that erythropoietin is part of the normal mechanism controlling erythropoiesis.

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Received May 26, 1960. P.S.E.B.M., 1960, v104.

Direct Measurement of Radioactive Sulfate Uptake by Rat Mast Cells.* (25913)

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The *in vivo* uptake of $S^{35}O_4$ by an organic component of mast cells has been demonstrated using autoradiography. By means of intensity grading(1) or grain counting(2) an estimate of rate of uptake and loss of radioactivity was achieved. The grading method is at best semi-quantitative, while the grain counting technic is cumbersome. Therefore we searched for a quantitative method of reasonable simplicity. Millipore filters have proved useful in several types of cytologic study. Collection of free cells on these filters has been employed in clinical cancer cytology (3,4), in *in vitro* studies of cell function(5) and in tissue culture(6). This study was undertaken to extend this method of cell collection to permit direct measurement of incorporation of radioactively labeled substances into cells. The cells chosen for the investigation were free peritoneal mast cells of the rat. Radioacitve sulfate was used as the tracer compound, since sulfate is known to be incorporated into mast cell heparin(7).

Materials and methods. Isotope labeled sulfate obtained as $H_2S^{35}O_4$ in dilute HCl was titrated to neutrality with NaOH and diluted to the desired concentration with .8% aqueous NaCl. This solution was administered intravenously in the tail of an etherized rat. Sprague-Dawley male rats weighing 280-390 g were used in most experiments. Mast cells were obtained from rat peritoneal cavities by the procedure previously described(8). Separation of mast cells from other peritoneal cells was achieved by centrifugation through

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* This work supported in part by grant from U.S.P.H.S.

† Work performed as U. S. Public Health Service Post-doctoral Research Fellow of Nat. Heart Inst.

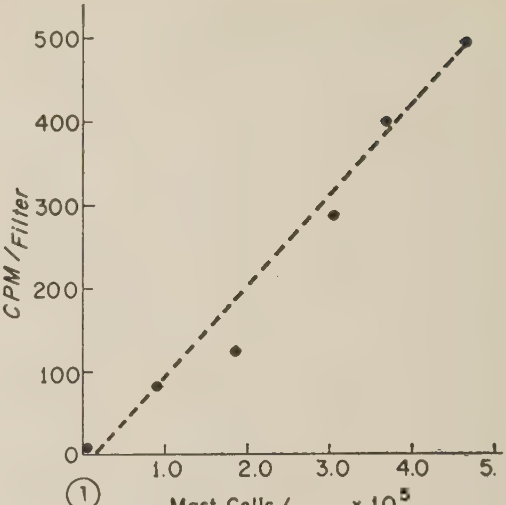
‡ Medical Student Summer Trainee in Exp. Pathology, present address: Johns Hopkins School of Med., Baltimore, Md.

concentrated sucrose solution(9). Samples of the cells in the initial peritoneal washings as well as the separated cell fractions were collected on Millipore filters, Type AA, 1 inch diameter, using the standard chimney assembly (purchased from Millipore Filter Corp., Watertown, Mass). The cells were washed with Tyrode's solution and the filters allowed to dry on the absorbent pads supplied with the filters. A glass slide placed over the filter prevented curling during drying. The Millipore filter on the pad was then placed directly in a 1 inch inner diameter planchette and the radioactivity measured. A GM tube with a "micromil" window (Nuclear, Chicago) was used. Counting rates were recorded in counts per minute (CPM) and were corrected for background. No corrections for isotope decay were necessary since all measurements within a single experiment were made on the same day. Counting rates were low enough to obviate a correction for resolving time. A small number of autoradiographs were prepared by the coating emulsion technic. Following 4% formaldehyde fixation, the filters were dried between 2 glass slides, attached to a slide with nitrocellulose and coated with NTB-3 emulsion (generously supplied by Eastman Kodak Co., Rochester, N. Y.). The emulsion was then exposed for 14 days at 4°C and developed in Kodak Dektol. The filters were stained with .1% aqueous safranin O, dehydrated through alcohol, cleared in xylene and mounted in permount.

Results. Examination of toluidine blue stained Millipore filter preparations revealed the mast cells and other cells to be distributed in a single layer on the filter. The conditions of counting therefore were those of a very thin layer, uniformity of the layer depending on cell thickness. In order to test the feasibility of direct counting on the filter, counts per minute were measured for varying numbers of $S^{35}O_4$ containing mast cells from the same animal. The data from this experiment are presented in Fig. 1. The coefficient of correlation of numbers of cells with CPM was + .98, indicating linearity within the range of the numbers of cells used.

A study of the relationship of administered dose of $S^{35}O_4$ to radioactivity incorporated into the mast cells provided a further test of the method. A linear relation was obtained (Fig. 2).

Since the first 2 experiments were conducted with complete peritoneal cell washings it was



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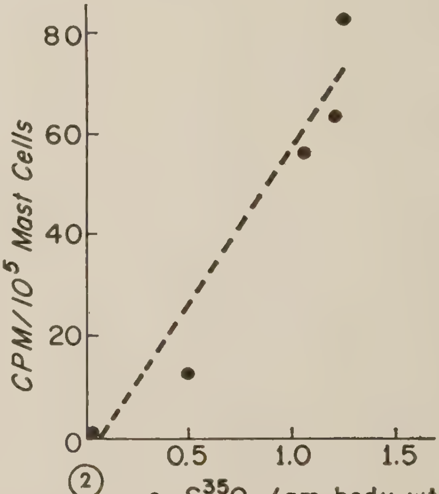

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FIG. 1. Relationship between CPM/Filter and No. of mast cells/filter. Initial peritoneal washing from one animal was diluted to give varying numbers of $S^{35}O_4$ labeled mast cells. Two filters were prepared at each dilution and counting was carried out for 3 min.

FIG. 2. Effect of dose of $S^{35}O_4$ administered on CPM/10^5 mast cells 24 hr after I.V. administration. Each point represents avg of counts on 2 filters prepared from one animal. Counting was carried out for 3 min.

of interest to know whether or not all the radioactive label was present in the mast cells and further to examine the rate of incorporation of $S^{35}O_4$ into mast cells. Ten rats were given 1.25 μc/g body wt. of $S^{35}O_4$ intravenously, and at each of the times, 2, 4, 16, 48 and 168 hours following administration 2 animals were sacrificed. Radioactivity of the whole cell population, the mast cell fraction and the interface mixed cell fraction was measured on 2 samples from each of the animals. Mast cells comprised, by number, 5 ± 2% of the initial peritoneal washings, 94 ± 4% of the mast cell fraction (MC Fraction) and 1 ± 0.3% of the other peritoneal cell fraction (OPC Fraction). Since neither the MC fraction nor the OPC fraction constituted a uniform cell population, the values for CPM per 10^5 mast cells $\frac{\text{CPM}}{10^5\ \text{MC}}$ and CPM per 10^5 other peritoneal cells $\frac{\text{CPM}}{10^5\ \text{OPC}}$ were derived from the solution of 2 simultaneous equations:

$$1.\ \frac{\text{CPM}}{10^5\ \text{OPC}} \cdot \frac{\text{OPC} \times 10^5}{\text{Filter}} + \frac{\text{CPM}}{10^5\ \text{MC}} \cdot \frac{\text{MC} \times 10^5}{\text{Filter}} = \frac{\text{CPM}_{\text{MC Fraction}}}{\text{Filter}}$$

$$2.\ \frac{\text{CPM}}{10^5\ \text{OPC}} \cdot \frac{\text{OPC} \times 10^5}{\text{Filter}} + \frac{\text{CPM}}{10^5\ \text{MC}} \cdot \frac{\text{MC} \times 10^5}{\text{Filter}} = \frac{\text{CPM}_{\text{OPC Fraction}}}{\text{Filter}}$$

Data for radioactivity of the mast cells and the other peritoneal cells are presented graphically in Fig. 3. By 24 hours radioactivity of the mast cells accounted for 89% of the radioactivity in the total peritoneal cell population.

The internal consistency of the values obtained for the $\frac{\text{CPM}}{10^5}$ MC Fraction and $\frac{\text{CPM}}{10^5}$ OPC Fraction was established by multiplying these values by the respective numbers of cells in the initial washings. The sums of these two products, representing the total $\frac{\text{CPM}}{\text{Filter}}$ expected, were compared to the values obtained from direct measurements of filters prepared from the initial washings. Close agreement was observed as seen in Table I.

TABLE I. Comparison of Expected and Obtained Radioactivity of the Initial Washings.

Time, hr	CPM* Expected	CPM* Found
2	113	125
4	106	107
16	100	114
48	182	168
168	199	177

* Avg values from 2 animals with 2 filters counted on each animal.

An analysis of the errors of measurement was made. When CPM for 27 pairs of duplicate filters were compared, the mean deviation from the means of the pairs was 9.1% with a standard deviation of ± 7%. This variation is accounted for by errors in pipette measurements of aliquots delivered to the filters and the probable lack of uniform distribution of cells in the solution. This lat-

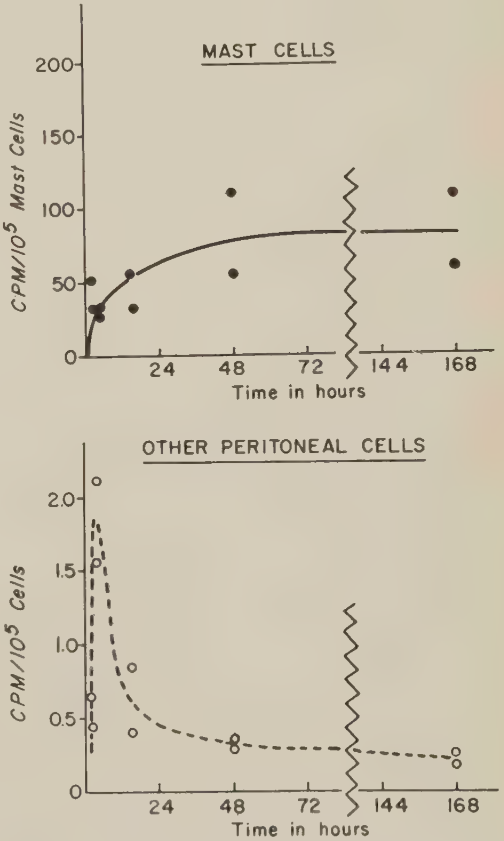

FIG. 3. Incorporation of $S^{35}O_4$ in peritoneal cells. Each point represents the avg of counts on 2 filters prepared from one animal. Time required to accumulate 1000 counts was measured.

ter factor is of particular significance in the viscous sucrose solutions. The larger variation obtained when results from 2 animals were compared (mean deviation from the mean of a pair was 20% with a standard deviation of ± 10% for 10 pairs) was presumably a function of biological variation and variations in cell counts. This latter error is remediable by counting a greater number of cells.

Discussion. Recent evidence has confirmed the fact that heparin is the sulfated mucopolysaccharide which confers on mast cells their characteristic metachromasia(7,10,11). *In vitro* experiments with a serially propagated mouse mast cell tumor have elucidated in part the mechanism of incorporation of sulfate into mast cell heparin(12). Though it is probable that most of the sulfate incorporated into mast cells in our studies is located in the heparin, definite proof of the chemical site of incorporation requires extraction and identification of the $S^{35}O_4$ containing substance(s).

Our experiments indicate that peritoneal mast cells do incorporate intravenously administered sulfate and that the loss of the sulfate is sufficiently low to be indiscernible within a period of 7 days. Guidotti(2) employing autoradiography in a study of SO_4 metabolism by tissue mast cells, found a 50% loss of activity on the basis of track counts in adult rat abdominal skin mast cells in the period between 48 and 144 hours after a single I.V. dose of $S^{35}O_4$. Our data are too scant to assert categorically a significant difference from Guidotti's results. The apparent discrepancy between his results and those of Jorpes *et al.*(1) is similarly in question. Further quantitative data with careful control of such parameters as site of origin of mast cells, and age and sex of rats employed are required to resolve the problem.

The Millipore filter method for direct determination of mast cell radioactivity should permit study of rate of incorporation and turnover of sulfate in the cells under various conditions known or postulated to affect mast cells such as antigen-antibody reactions, 48/80 treatment, hormonal excess or deficiency, and aging. The method provides a measure of mast cell activity quite different from changes in number, morphology and amine content previously relied on for information in elucidating the function of mast cells.

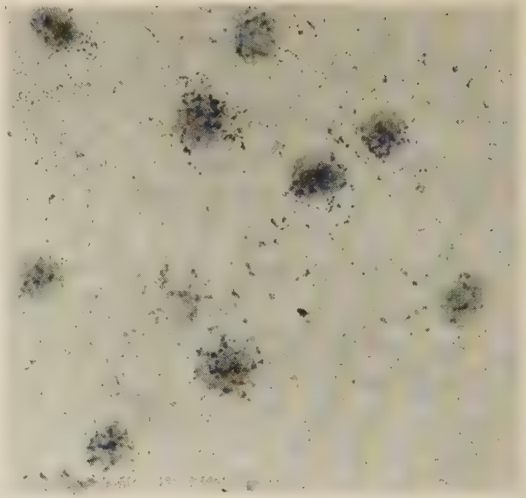

FIG. 4. Autoradiograph at 24 hr of peritoneal cells, prepared by coating emulsion technic. 14 days exposure at 4°C. Stained with .1% Safranin O. The large stained cells are the mast cells; the other cells appear only as faint ghosts.

The significance of the presence of $S^{35}O_4$ in the peritoneal cells other than mast cells 4 hours after administration of the sulfate is not known. Since this fraction is diverse, including neutrophils, eosinophils and lymphocytes, it may be that only one of these cell types is responsible for the radioactivity of this fraction. Careful autoradiography should reveal any non-uniform distribution of the $S^{35}O_4$ in these cells.

The method of direct counting of a monolayer of radioactively labeled cells on Millipore filters in conjunction with cell separation technics has proved applicable to study of sulfate incorporation by mast cells. The method is potentially useful for studies of other cells found in suspension normally or separated from tissues or tissue culture outgrowths with agents such as trypsin and ethylenediaminetetraacetic acid. The application of the Millipore method of collecting mast cells has been reported in an examination of the mechanism of degranulation(13).

Summary. 1) Radioactivity of $S^{35}O_4$ labeled cells can be measured directly with cells collected on Millipore filters. 2) Following a single intravenous injection of $S^{35}O_4$ rat peritoneal mast cells take up the radioactivity; the maximum is reached by 48 hours. No loss of radioactivity is evident in the following 5 days. Radioactivity in other free cells of peritoneal fluid attains a maximum at 4 hours and then falls rapidly to low levels.

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Received May 27, 1960. P.S.E.B.M., 1960, v104.

Local Shwartzman Reaction in Rabbit Oral Mucosa with Endotoxin from Oral Bacteria.* (25914)

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(Introduced by I. Zipkin)
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Although endotoxins from many bacteria have been isolated and tested for toxicity, little work has been devoted to endotoxins from oral microorganisms. Recently, endotoxins from oral bacteria, isolated by tryptic digestion of cells and also by the phenol-water extraction method, were shown to possess many chemical properties and biological activities characteristic of classical lipopolysaccharide endotoxins(1,2). Primary toxicity to rabbit skin was demonstrated, and the localized Shwartzman reaction of hemorrhagic necrosis was produced by administering these endotoxic preparations first locally into skin, and then intravenously. The local Shwartzman effect has been achieved with many endotoxins in different tissues(3,4) yet no successful attempts seem to have been made to produce this reaction in tissues of the oral cavity. Because of the possibility that bacterial endotoxins may have etiologic significance in oral soft tissue diseases either through primary local toxicity or through Shwartzman-like effects, one of the recently isolated endotoxins was tested in rabbit oral mucous membrane.

Materials and methods. New Zealand white rabbits weighing 1.7 to 2.5 kg were anesthetized by intravenous injection of 40 mg per kg of pentobarbital and access to the posterior oral region was obtained by means of a rodent mouth prop previously described (5). With a modified 30 gauge needle, 50-100 μg (0.2 ml) of an endotoxin prepared by the phenol-water method from *Veillonella* organisms(1) was injected into an area which included the gingiva and palatal mucosa adjacent to the last 2 upper molar teeth. The corresponding site of the contralateral side was given a control injection either of 100 μg (0.2 ml) of soluble products from a human oral strain of viridans streptococcus or of 0.2 ml of 0.85% sodium chloride (saline). The streptococcal products contained proteins(6), carbohydrates (Molisch), and chloroform-soluble lipids, derived also by the phenol-water extraction procedure. Comparable dos-

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* The authors express thanks to Dr. Harold Stanley for aid in preparation of histological specimens and to John McGuire for preparing photomicrographs.

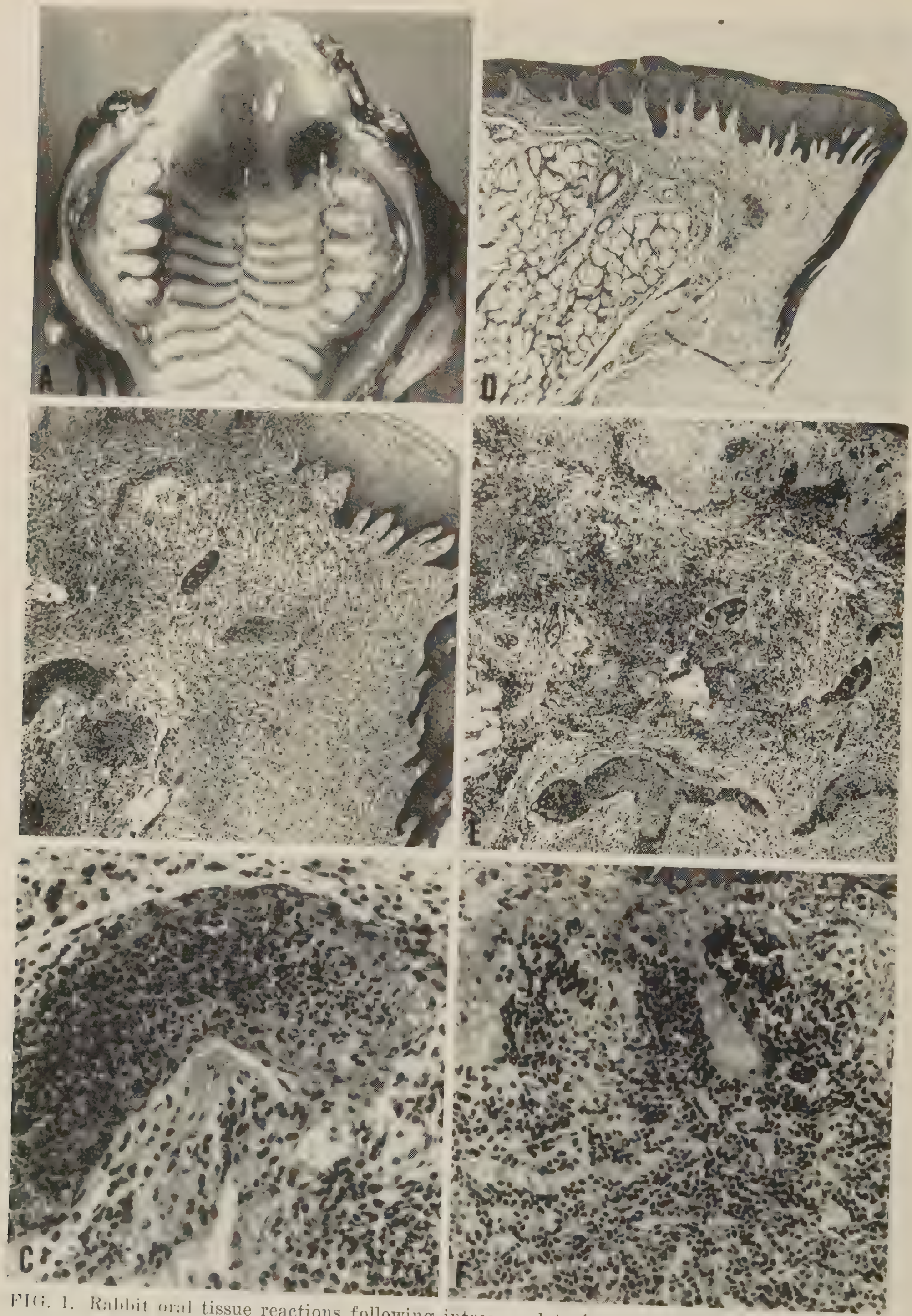

FIG. 1. Rabbit oral tissue reactions following intrav. endotoxin 14 hr after local inj. of *Veillonella* endotoxin and of streptococcal products.

A. Shwartzman reaction in gingivo-palatal mucosa of upper right jaw. Left side of jaw shows negligible reaction to streptococcal products.

B. Shwartzman reactive tissue stained to show diffuse and focal leucocyte infiltration and ages of these substances were also injected into the shaved skin of the animals. To test for the Shwartzman reaction, rabbits were given 125 μg of *Veillonella* endotoxin in the marginal ear vein 12-24 hours after local injection. Approximately 6 hours following the intravenous dose, animals were sacrificed, and tissue surrounding and including the molar teeth was fixed in formalin. Subsequently, the specimens were decalcified in 5% formic acid, imbedded in paraffin, and sectioned at 7 μ. In some instances, the soft tissue which included the lesion was dissected from bone and tooth and processed for histological examination without decalcification. Sections stained with hematoxylin and eosin, with Lillie's azure-eosin, and by the periodic acid-Schiff method were examined microscopically.

Results. In rabbits given preparatory and provoking injections of endotoxin, gross signs of the local Shwartzman reaction were evident. Dark red hemorrhagic areas became visible in the prepared sites within several hours following the intravenous dose, whereas control sites injected with streptococcal products showed barely perceptible discoloration (Fig. 1A). Histologically, the Shwartzman lesions showed the classical features previously described in skin(2,7,8,9) including extensive leucocyte infiltration (Fig. 1B), leucocytic thrombi (Fig. 1C), and hemorrhage (Fig. 1E,F). Control sites given streptococcal products and provoked with intravenous *Veillonella* endotoxin contained an occasional focus of necrosis with minimal leucocytic response and hemorrhage (Fig. 1D). In sites prepared with endotoxin but not provoked, the leucocytic response was considerably less severe than in the Shwartzman lesions and hemorrhage was absent. These unprovoked endotoxin lesions, however, contained much heavier leucocytic infiltration than either the provoked or the unprovoked lesions obtained with comparable amounts of streptococcal products. This difference in polymorphonuclear response was equally evident in both oral mucosa and skin. Comparison of response of oral mucosa and of skin to similar local injections or to similar combinations of local and intravenous injections revealed that gross and histologic manifestations following a specific type of insult were basically similar in both tissues.

Discussion. It has yet to be established that the laboratory demonstration of a local Shwartzman reaction in oral tissues by injection of endotoxin from organisms of the gingival crevice has a direct counterpart in human disease. However, the finding that a relatively temporary alteration in the blood can provoke a violent reaction in local sites within the oral cavity reflects the close relationship existing between oral tissue reactivity and systemic changes, and at least suggests that a similar mechanism may operate in oral disease. Periodontal conditions which should be considered in the light of the Shwartzman phenomenon include acute Vincent's necrotizing gingivitis and the acute exacerbations of chronic gingivitis frequently associated with an episode of generalized illness. In both of these acute conditions there is evidence of interplay between local tissue reactivity and somewhat temporary systemic alterations.

It is significant that the reaction of oral mucosa to injection of different substances resembles rather closely the skin reactions to these same substances. This finding gives support to use of skin as a convenient tissue for testing substances thought to be toxic to mucous membranes, and thus validates investigations of experimental infections in skin with oral bacteria(10,11).

Summary. The local Shwartzman reaction was elicited in the rabbit oral cavity and skin with endotoxin prepared by phenol-water ex-

several venous thrombi. Crevicular epithelium of upper right molar at right. H & E 50×.

C. High power view from B, showing numerous leucocytes in typical thrombus. H & E 209×.

D. Tissue adjacent to upper left molar showing small area of necrosis and minimal leucocytic response to local inj. of streptococcal products and intrav. endotoxin. H & E 51×.

E. Section from same block as B and C stained to show extravascular and intravascular distribution of red blood cells; white cells unstained. Alcian Blue-eosin B. 61×.

F. High power view of sub-epithelial area from E showing hemorrhage. Alcian Blue-eosin B. 238×.

traction procedure from a strain of oral *Veillonella*. Lipid-carbohydrate-protein products prepared by same procedure from viridans streptococci, or a solution of physiologic saline did not produce this reaction in either tissue. Lesions obtained in oral mucosa with each substance were grossly and histologically similar to lesions obtained with the same material in skin.

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Received May 31, 1960. P.S.E.B.M., 1960, v104.

Effect of Adrenalectomy and Cortisone on Eruption Rate of Incisors in Young Female Albino Rats.* (25915)

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Schour and Van Dyke(1) reported that rate of eruption of the continuously erupting rat incisor became progressively slower and finally ceased in hypophysectomized rats. Administration of growth hormone produced only a slight increase in rate of eruption of such animals but had no effect in intact rats. Baume *et al.*(2) observed that thyroidectomy in young rats produced a reduction in rate of eruption although not so severe as that produced by hypophysectomy. These authors (3) also reported that simultaneous administration of thyroxin and growth hormone in hypophysectomized rats brought about a restoration of the tissues of the incisor and an increase in rate of eruption. Schour and Rogoff(4) observed a disturbance in calcification of dentin in the rat incisor following adrenalectomy; however, due to the short survival period, rate of eruption was not measured. Leroy and Domm(5) reported that cortisone produced precocious eruption of incisors in young rats when administered to pregnant mothers, to the fetus *in utero*, or in newborn shortly after birth. Domm and Marzano(6) found that cortisone also accelerated rate of eruption of incisors when administered in sexually mature rats. Since adrenal function is depressed following hypophysectomy and since the rate of eruption of the rat incisor has been shown to be stimulated by adrenal cortical hormones we were interested in determining the effects of adrenalectomy and of cortisone and salt therapy on this rate in young female adrenalectomized rats.

Procedures. Twenty-four female rats, of the Sprague-Dawley strain, bilaterally adrenalectomized at 39 days of age, and 12 unoperated controls of the same age were studied. Twelve operated and 6 normals received daily subcutaneous injections of 1.5 mg of cortisone,‡ 6 days per week for 6 weeks. The remaining animals served as operated and intact controls. All controls received injections of physiological saline while adrenalectomized controls in addition received 1% NaCl in drinking water. At the end of the sixth week all treatment, daily injections and NaCl in drinking water was discontinued and observations continued for a second 6-week period.

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* This investigation supported in part by U.S.P.H.S. Research Grant.

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‡ Cortisone (Cortone Acetate) generously supplied by Merck, Sharp & Dohme, Division of Merck & Co., Philadelphia, Pa.

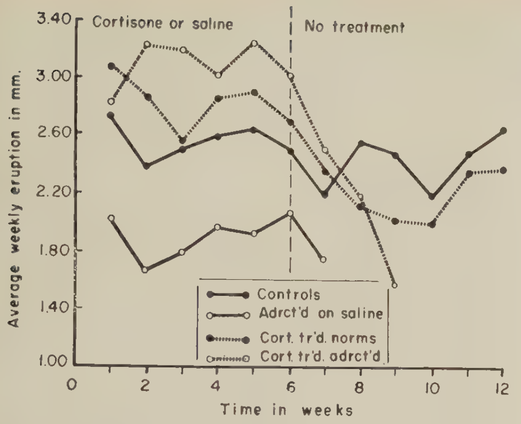

FIG. 1. Effect of adrenalectomy and cortisone on rate of eruption of maxillary incisors in young rats.

All animals were maintained on a diet of Purina Dog Chow pellets and water or water with NaCl *ad libitum*. Weekly eruption rates of upper incisors were determined by a modification of the method(2) previously described by Schour and Van Dyke(1). Eruption rates of adrenalectomized rats surviving throughout the 6-week period following cessation of maintenance treatment (3 cases) were eliminated from data and calculations. The t-test was employed to evaluate differences between the means of normal controls and each of the experimental groups, for each week, and for each of the 2 six-week periods. Differences were considered significant at the 5% level of probability.

Results. The results are shown graphically in Fig. 1. Eruption rates of adrenalectomized, saline-maintained, rats showed a significant decrease (–26%) by the end of the first week and a maximum decrease during the second (–30%) and third (–32%) weeks when compared with controls. During the fourth to sixth weeks there was a slight increase though the rates were still significantly below normal. Average weekly eruption rate for the first 6 weeks was 1.84 ± 0.34[§] mm (56 observations; P = .001), which was 28% below the average of controls, 2.56 ± 0.24 mm (36 observations) and highly significant. Following cessation of treatment eruption rates declined to the level observed during the first 3 weeks after operation. Average survival time following cessation of saline maintenance in adrenalectomized animals was 6.4 ± 3.2 days, with only one animal surviving the remainder of the second period in apparent good health. Body weights of adrenalectomized rats showed weekly increases equal to those of normal controls up to the third week but thereafter weekly increases were less pronounced so that by the end of the sixth week of treatment body weights were 11% below those of normal intact rats. Following cessation of treatment this difference increased to 20% as the animals succumbed of adrenal insufficiency.

Cortisone produced an increase in rate of eruption of adrenalectomized rats which was statistically significant by the second week and continued so for the remainder of the 6-week period. Average rate of eruption for the period of cortisone treatment was 3.08 ± 0.38 mm (60 observations; P = .001) which was 21% greater than that of untreated controls and highly significant. Cessation of treatment was followed by a sharp decline in rate of eruption. Average survival time following cortisone withdrawal was 22.1 ± 5.3 days with 2 rats surviving the remainder of the second 6-week period. Cortisone administration produced a severe reduction in body weight so that by the end of the first 6-week period adrenalectomized rats were 40% below the average for untreated intact controls and 29% below the average of untreated adrenalectomized controls.

Cortisone produced a slight increase in rate of eruption of intact rats which proved to be statistically significant only during the second week (+20%). Average eruption rate for the period of treatment was 2.82 ± 0.30 mm (36 observations; P = <.2) which was 10% greater than that of untreated controls but not significant. Following cessation of treatment eruption rates declined below those of normal controls and continued at a slightly reduced rate for the remainder of the second 6-week period. This reduction was found to be statistically significant during the 8th, 9th and 12th weeks. However, average rate for the second 6-week period did not differ significantly from the average for controls; 2.21

[§] Standard deviation.

± 0.32 mm (36 observations; $P = >.1$) compared with 2.46 ± 0.27 mm (36 observations) for controls. Cortisone administration resulted in a reduction of body weight to 14% below that of untreated controls by the end of 6 weeks which was quickly regained following cessation of treatment. There was no significant difference at autopsy, 6 weeks following cessation of treatment, between weights of the adrenals of treated and control rats.

Discussion. It has been reported(1,3) that hypophysectomy in the rat produces a severe retardation in rate of eruption of the incisors and that growth hormone is ineffective in restoring the normal rate. The reduction in rate of eruption observed following thyroidectomy in the rat is not so severe as that reported for hypophysectomy(3). This would indicate that factors other than absence of thyroid hormone may be involved. The depression observed in eruption rate following adrenalectomy and the stimulation in rate noted following cortisone administration indicates that the adrenal must be considered in any theory involving a hormonal control of incisor eruption rate. Whether the cortisone-induced acceleration in eruption is due to a direct stimulation of the incisor, as has been reported for thyroxin(3), or whether it is due to some other factor or factors remains to be determined.

Summary. Adrenalectomy in young female rats resulted in a significant reduction in rate of eruption of incisors which averaged 28% below that of unoperated controls during 6 weeks of measurement. Daily administration of cortisone produced a significant acceleration in rates of adrenalectomized rats to levels above that of nontreated intact controls. It is suggested that the severe reduction in rate of eruption of the continuously erupting incisor observed by others following hypophysectomy is due to loss of effect of both thyroid and adrenal hormones.

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Received May 31, 1960. P.S.E.B.M., 1960, v104.

Specificity of Allergic Reactions. II. Azoproteins in the Anamnestic Response. (25916)

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Delayed and Arthus hypersensitivities differ in their immunologic specificity(1,2). Guinea pigs sensitized with 5-15 μg of a conjugate of picryl-hen egg albumin in Freund's adjuvant (without mycobacteria) develop delayed hypersensitivity and ultimately circulating antibody to the whole conjugate or the protein moiety, but only circulating antibody to the hapten. Delayed hypersensitivity can differentiate widely different antigens, such as hen egg albumin from bovine gamma globulin, but cannot readily distinguish a conjugated protein from the same unconjugated protein. Antibodies can differentiate between minor chemical differences in antigens(3). For example, an antigen composed of a protein diazotized to one acid radical, when injected into animals, formed antibodies which did not cross-react with the same protein diazotized to a different acid radical. An immune serum to para-aminobenzoic acid-horse serum formed precipitates with the homologous antigen, but not with horse serum conjugated to the heterologous haptens such as para-sulfanilic acid, para-arsanilic acid, or aniline radicals(4). Antibody even differentiated between ortho, meta, or para position of a carboxyl radical on a benzene ring. In previous experiments(2,5), an anamnestic effect was produced in guinea pigs after a primary injection of a homologous protein in quantities sufficient to produce delayed hypersensitivity but not circulating antibody and after a subsequent injection of hapten-homologous protein conjugate. In animals given a primary injection of a homologous hapten-heterologous protein, an anamnestic response was not detected on subsequent introduction of conjugate. The haptens studied were picryl chloride and 1, fluoro, 2-4 dinitrobenzene; the proteins, purified hen egg albumin and bovine gamma globulin. Results obtained with various combinations of proteins and haptens indicated that the basic protein might be modified (denatured) to varying extents depending upon the hapten. Therefore, to obtain information on relative specificities of delayed and Arthus reactions with more uniformly conjugated proteins, further experiments were conducted with antigens composed of specific diazotized radicals coupled to purified crystalline proteins. The present paper is concerned primarily with anamnestic responses wherein induction of delayed hypersensitivity to a homologous protein or azoprotein prior to a secondary injection of azoprotein plus a hapten caused enhancement of antibody formation to hapten.

Materials and methods. Animals. Guinea pigs of the Hartley strain weighing 400 to 450 g were used for studies on hypersensitivity. White or albino guinea pigs not necessarily of the Hartley strain weighing 300 to 400 g were employed in the passive cutaneous anaphylaxis (PCA) tests for determination of antibody in test sera. Once guinea pigs had been skin tested, they were not used again. *Antigens.* Proteins were prepared as described previously(2). Diazotization and coupling of proteins were carried out with modifications of basic methods previously described(4,6). As an example, the method used for coupling of para-aminobenzoic acid (PABA) with hen egg albumin (HEA) is given. Other simple chemicals used for coupling were employed in quantities equivalent to that of the PABA. 1.37 g (0.01 Mole) of PABA were dissolved in 150 ml of distilled water and 26 ml (0.026 Mole) of N HCl. Diazotization was carried out with 0.011 M sodium nitrite at a temperature of 0°C. Diazotized protein was then precipitated by acidification to litmus paper with 10% trichloroacetic acid. The precipitate was centrifuged, resuspended in 19 ml saline, and dissolved with 0.1N NaOH. Seven additional reprecipitations were carried out. Finally, the precipitate was dissolved in 20 ml saline adjusted to pH 7.0 with 0.1N NaOH. Other preparations were treated in a similar manner. Thus, aniline (An), p-aminosulfonic acid (PASA), p-aminoarsonic acid (PAAA) or m-aminobenzoic acid (MABA) were diazotized and coupled to HEA, bovine gamma globulin (BGG), human gamma globulin (HuGG), or bovine plasma albumin (BPA). *Sensitization.* Antigens were dissolved either in physiologic saline plus 1% guinea pig serum or in Freund's adjuvant (Difco) without mycobacteria, and a total volume of 0.5 ml injected intracutaneously into the digits of the feet. *Skin tests.* Guinea pigs were tested on the flanks by intracutaneous injection of 0.1 ml of a solution containing 50 μg protein/ml saline. Reactions were observed and diameters of areas of induration measured at intervals up to 4 hours after sensitization and between 18 and 24 hours. *Antibody determination.* Guinea pigs were bled prior to being skin tested, and the resulting sera assayed for antibody by the passive cutaneous anaphylaxis (PCA) reaction(7). By this technic, as little as 0.002-0.003 μg AbN can be detected. In these tests, 0.1 ml antiserum was injected intradermally in the back or sides. About 3 hours later, 350 μg protein in 0.5 ml physiologic saline and 0.5 ml 1% Evans blue in physiologic saline were administered intravenously. Fifteen to 30 minutes later, areas of blueing pigmentation were measured and recorded.

Results. Hypersensitivity to proteins coupled to diazotized acid radicals. Guinea pigs were sensitized in the footpads with 15 μg of hen egg albumin (HEA), hen egg albumin

TABLE I. Cross-Reactions in Guinea Pigs Sensitized with 15.0 μg PABA · HEA in Adjuvant.*

No. of guinea pigs	Day tested	Skin tested with 5 μg																							
		PABA · HEA			PAAA · HEA			PASA · HEA	An · HEA			HEA			BGG			BPA	An · BGG		PABA · BGG			PABA · BPA	PAAA · BGG
		A	Ab	D	A	Ab	D	Ab	A	Ab	D	A	Ab	D	A	Ab	D	Ab	A	D	A	Ab	D	Ab	Ab
2	5		N	++						N	0		N	+++			0			0			0		
3	7		N	"						N	±		N	++			0			0			0		
5	8		N	"	0	N	+			N	++	0	N	"			0			0			0		
5	9		N	+	0	N	+	N	0	N	±	0	N	+	0	N	0		0	0	0		0		N
3	10	++	⅓	++	0	N	±					0	N	+	0	N	0	N			++	⅓	0	⅓	N
3	11	+++	P		0	N	"					0	N	+	0	N	0	N			+++	P	†	P	N
5	12	"	P			⅕		N	0	N	++	0	N	++	0	N	0	N	0	0	"	P	†	P	N
2	15	"	P			N		N	0	N	"	0	N	±	0	N	0	N	0	0	"	P	†	P	N
2	20	"	P			½		N	0	N	±	0	N	+	0	N	0	N	0	0	"	P	†	P	N

* Similar results obtained after sensitization with 5 μg PABA · HEA in adjuvant.

† Reaction not determinable.

Numerator of fraction indicates No. of guinea pigs showing antibody by PCA test. Difference between denominator and numerator indicates animals possibly showing delayed hypersensitivity.

A, Arthus-type hypersensitivity.
Ab, Circulating antibody (by PCA test(4)).
D, Delayed hypersensitivity.

P, Circulating antibody present; N, circulating antibody not detected.
0, Mean diameter of induration <10 mm (in animals showing particular reaction).

±,	Mean diameter of induration about	10 mm.
+,	*Idem*	11 to 14 mm.
++,	"	15 to 19 " .
+++,	"	20 to 24 " .

coupled to diazotized p-aminobenzoic acid (PABA · HEA), to p-aminoarsonic acid (PAAA · HEA), or to p-aminosulfonic acid (PASA · HEA), bovine gamma globulin (BGG), or bovine gamma globulin coupled to diazotized p-aminobenzoic acid (PABA · BGG) in Freund's adjuvant without mycobacteria. In animals sensitized with 15 μg HEA in Freund's adjuvant, delayed hypersensitivity could be detected not only to HEA itself but also to any of the HEA conjugates, such as HEA coupled to diazotized (a) aniline (An · HEA), (b) p-aminosulfonic acid (PASA · HEA), (c) p-aminoarsonic acid (PAAA · HEA) or (d) p-aminobenzoic acid (PABA · HEA). Delayed hypersensitivity could not be discerned in any of the guinea pigs skin-tested with the foregoing diazotized radicals coupled to heterologous proteins such as BGG, human gamma globulin (HuGG), or bovine plasma albumin (BPA). However, from the 9th to 20th day, when Arthus type hypersensitivity and circulating antibody could be detected, reactions occurred only with the homologous HEA, and not with any of its conjugates. Antibody to a closely related conjugate such as An · HEA could not be detected.

Sensitization of guinea pigs with PABA · HEA resulted in delayed hypersensitivity from the 5th to at least the 10th day (Table I). This hypersensitivity was directed only against HEA and conjugates containing HEA. On the 10th day, following appearance of circulating antibody and Arthus reactions, specificity was no longer directed against the protein but against the hapten PABA conjugated to any of the proteins (HEA, BGG, BPA). Two of the 30 sera from these animals did cross-react with PAAA · HEA in the PCA test. Since, however, they did not react with PAAA · BGG, their specificity was presumably directed toward the HEA portion of the conjugate.

Similar results were obtained in 38 guinea pigs sensitized with PASA · HEA, 23 sensitized with PAAA · HEA, and 33 sensitized with PABA · BGG. In all cases, the animals first showed delayed hypersensitivity to any conjugate containing the homologous protein

TABLE II. Anamnestic Response to PABA · HEA in Guinea Pigs Administered a Primary Dose of 1 μg PABA · HEA in Saline and a Secondary of 5 μg PABA · HEA in Adjuvant. (10 days between inj.)

		Detectable antibody by PCA test						
		Antigen						
No. of guinea pigs	Day tested	PABA · HEA	PABA · BGG	PABA · BPA	MABA · HEA	MABA · BGG	PAAA · HEA	PAAA · BGG
4	4	N	N	N	N	N	N	N
4	5	1/4	N	N	N		N	N
4	6	P	3/4	P	2/3	2/3	N	N
4	7	3/4	3/4	3/4	3/4	3/4	N	N
4	8	P	P	2/4	"	2/4	N	N
4	9	P	3/4	3/4	"	"	N	N
4	10	P	P	P	P	3/4	N	N
4	11	P	P	P	P	"	2/4	N
4	12	P	P	P	P	"	N	N
3	14	P	P	P	P	P	2/3	N
3	16	P	P	P	P	2/3	N	N
3	18	P	P	P	P	P	2/3	N

See Table I for legend.

and then Arthus hypersensitivity to any conjugate containing the homologous hapten. Sensitization with these conjugates further indicated (a) that PASA · HEA was a weaker antigen than the others in that Arthus reactions did not appear until the 12th to 13th day after sensitization and areas of induration and erythema were smaller; and (b) that PAAA · HEA induced delayed hypersensitivity to HEA and to its conjugates PABA · HEA and PASA · HEA up to the last skin tests on the 16th day, although Arthus reactions appeared on the 10th day to PAAA coupled with HEA, BGG, or HuGG.

Azoproteins in an anamnestic response. A primary injection of 1.0 μg PABA · HEA in serum-saline was administered in the footpads of guinea pigs. This dosage was used since typical delayed hypersensitivity develops, but circulating antibody can not be detected by PCA tests. Ten days later, the animals were injected with 5.0 μg PABA · HEA in Freund's adjuvant (Table II). On the 5th to 6th day after secondary sensitization, antibody to the homologous PABA · HEA was detected. In contrast, animals given a single injection of 5.0 to 15.0 μg PABA · HEA in adjuvant usually develop antibodies on the 10th or 11th day (Table II). The antibody formed to PABA · HEA in animals given a secondary stimulation also reacted with PABA · BGG and PABA · BPA, but not with An · HEA, PASA · HEA or HEA. Six sera reacted with PAAA · HEA, but not with heterologous protein conjugate PAAA · BGG. The cross-reaction was apparently associated with the protein portion of the conjugate or with the conjugate as a unit.

Guinea pigs sensitized with a primary dose of An · HEA, HEA, or PAAA · HEA, sufficient to develop delayed reactions but not detectable antibody, produced an anamnestic response to a subsequent injection of PABA · HEA (Table III). Delayed hypersensitivity to HEA after primary injection must have served as a basis for the anamnestic response, since the protein was the only common antigenic factor in 2 injections. The capacity of homologous protein to induce an anamnestic effect was particularly significant in that enhancement occurred in rate of antibody production against a hapten (PABA) which was not contained in the primary injection.

When secondary stimulation with PABA · HEA was preceded by injection of a homologous hapten-heterologous protein (PABA · BGG), an anamnestic response did not follow (Table III). Antibody to PABA · HEA appeared at the same interval after the secondary injection as did antibody in control animals that received only a single injection of antigen. Reintroduction of hapten on a heterologous protein therefore did not accelerate antibody response to the hapten.

Discussion. In previous work(1,2), haptens such as picryl chloride were connected

TABLE III. Anamnestic Response to PABA · HEA and Variants. (10 days between inj.)

Antigen		No. of guinea pigs	Day tested	Detectable antibody Antigen					
Primary	Secondary			PABA· HEA	PABA· BGG	PAAA· HEA	PAAA· BGG	An· HEA	HEA
1 μg An·HEA in saline	5 μg PABA· HEA in adjuvant	3	6	N	N	N	N	N	N
		3	7	⅔	⅓	N	N	N	N
		3	8	P	P	N	N	N	N
		3	9	P	P	N	N	N	N
		3	17	P	P	⅔	N	N	N
		3	20	P	P	N	N	N	N
1 μg HEA in saline	*Idem*	3	6	N	N	N	N	N	N
		3	7	⅓	N	N	N	N	N
		3	8	P	⅓	N	N	N	N
		3	9	P	P	N	N	N	N
		3	10	P	P	N	N	N	N
		3	20	P	P	⅔	N	N	N
		3	22	P	P	N	N	N	N
1 μg PAAA· HEA in saline	"	3	7	N	N	N	N		N
		3	8	P	P	N	N		N
		3	9	P	P	N	N		N
		3	11	P	P	⅓	⅓		N
		3	12	P	P	"	"		N
		3	17	P	P	⅔	⅔		N
		3	25	P	P	⅓	N		N
		3	27	P	P	⅔	⅓		N
1 μg PABA· BGG in saline	"	4	7	0	0				
		4	8	0	0				
		4	9	¼	0				
		4	10	"	¼				
		4	11	"	"				
		4	12	P	P				
		4	14	P	P				

See Table I for legend.

directly to proteins. Each hapten is probably different in its chemical reactivity and therefore, in the process of conjugation, could have altered the basic nature of the protein in a different way. To minimize the differences produced in the protein molecule by conjugation, haptens were first diazotized and the protein conjugated under similar chemical environments. Comparisons in immunologic activity of haptens could then be made with the knowledge that what differences existed were probably due to the hapten and not to modifications in the basic protein molecule.

The observation to be emphasized concerns accelerated production of antibody to the hapten of a conjugated protein when immunization is preceded by injection of the protein alone. This phenomenon can best be explained by the assumption that an early step in antibody production is a state of delayed hypersensitivity to the protein as a whole, and that this step is superseded by production of circulating antibody if enough antigen is present or if a booster dose is administered. The specificity of the circulating antibody is, however, directed toward small chemical configurations which may be present only in the booster antigen.

Earlier observations suggested the possibility that delayed hypersensitivity might represent an early stage in production of circulating antibody(8). Administration of egg white or horse serum to tuberculous guinea pigs produced a state of hypersensitivity characterized by delayed and prolonged skin reactions, absence of demonstrable antibodies in the blood stream, failure of passive transfer with serum, and insusceptibility to anaphylactic shock after intravenous injection of the specific antigen. Others(9-12) subsequently noted that repeated skin-testing of man or animals caused the subjects to develop a 24-hour inflammatory reaction of the delayed type, which, when the series of intradermal injections were continued, was replaced by a hypersensitivity of the early type. Histological evidence has also indicated(13,14) that early in the course of immunization a delayed reaction develops characterized by mononuclear cells and absence of serum antibody, but that maturation of mononuclear cells to plasma cells occurs simultaneously with detection of circulating antibody.

Delayed allergy was further implicated as a possible phase in the immune process when a single intradermal injection of a minute quantity of highly purified soluble protein (*e.g.*, 1 Lf diphtheria toxoid) in Freund's adjuvant was found to induce typical delayed reactions. Antitoxin could not be detected by the rabbit intracutaneous test, which can detect 0.0024 μg AbN, and hypersensitivity could be transferred passively by washed lymph node cells(15). Duration of delayed hypersensitivity varied with amount and type of antigen, but was eventually masked by the appearance of circulating antibody and the concomitant Arthus reaction. However, if the amount of antigen injected is lowered sufficiently, delayed hypersensitivity may be produced which is not masked by circulating antibody(5). Evidence that the delayed reaction may be the basis for the anamnestic response has also been presented((15,16,5).

Studies of immune reactions in guinea pigs sensitized with conjugated proteins revealed the specificity of delayed allergy to be associated with the protein portion of the molecule, while specificity of the circulating antibody was directed primarily toward the hapten(1,2). If delayed hypersensitivity is an early stage in antibody production, then delayed hypersensitivity should precede detection of antibody in the circulation. The failure to observe delayed hypersensitivity to haptens prior to formation of antibody appears at first to indicate that delayed and Arthus type hypersensitivities are independent reactions, since antibody is formed to a chemical group (the hapten) without previous induction of a delayed hypersensitive state. If delayed hypersensitivity to the protein and antibody production to the hapten are completely unrelated, the 2 phenomena should have no effect on one another. The foregoing data, however, indicate that there is an intimate relation between delayed hypersensitivity to the protein portion and Arthus hypersensitivity to the haptenic portion of a conjugated protein. When an animal is in a state of delayed hypersensitivity to a protein, the immunological mechanism is primed to produce antibody to antigenically specific groups on that protein or to haptens attached to it. The primed mechanism responds to the second stimulus of the protein, but the resulting antibody is apparently specific for certain particular loci on the protein surface. When, therefore, HEA, An • HEA, or PAAA • HEA is used as the primary sensitizing antigen, the primed cell responds anamnestically to reintroduction of the homologous protein. If a heterologous hapten such as PABA is attached to HEA, response to the hapten is simultaneously accelerated.

In experiments involving anamnestic response, in which a protein was used as a primary dose and a homologous protein conjugate as the secondary dose to cause a typical anamnestic response to the hapten, occasional sera reacted with a heterologous hapten-homologous protein conjugate (Tables II and III). When a conjugate with the same heterologous hapten but a heterologous protein was assayed with these sera, no reaction occurred. Also, the homologous protein by itself failed to react with the antiserum. Hence, the antibody is probably being oriented toward a modified protein, such as may exist in the conjugate, or more likely toward the whole conjugate.

Summary. Injection of 15 μg of a hapten-azoprotein, such as p-aminobenzoic acid diazotized to hen egg albumin, in Freund's adjuvant, into footpads of guinea pigs produces delayed hypersensitivity in 5 days and circulating antibody in 10-11 days after sensitization. Injection of 1 μg of such conjugate in saline produces delayed hypersensitivity but no detectable circulating antibody. Specificity of delayed hypersensitivity is oriented toward the protein; that of circulating antibody, toward the hapten. When a primary dose of 1 μg in saline of protein, azoprotein or

heterologous hapten-homologous protein is followed by a secondary dose of hapten-homologous protein, antibody response to the hapten is accelerated. A primary sensitizing dose of hapten, such as p-aminobenzoic acid (PABA) + protein such as hen egg albumin (HEA), followed by a secondary dose of the same hapten attached to heterologous protein, does not result in accelerated response to the hapten. These phenomena are explained if delayed hypersensitivity is considered an early step in antibody synthesis.

Technical assistance of Jane Nishio, Aspascia Cobure, and Leroy F. Peel is gratefully acknowledged.

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Received June 1, 1960. P.S.E.B.M., 1960, v104.

Antigenicity of Connective Tissue Extracts II. Stimulation of Auto- and Iso-antibodies by Heterologous Antigen.* (25917)

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Previous studies in our laboratory(1) have shown that saline extracts from rabbit tendons combined with Freund's adjuvant are antigenic to guinea pigs. Circulating complement fixing (C.F.) and precipitating antibodies to these connective tissue extracts could be demonstrated(1). Growth of the majority of the guinea pigs injected subcutaneously with extracts of rabbit tendon was markedly diminished as compared to growth of control animals injected only with saline and adjuvant. This effect was not observed following injection of other tissue extracts (liver, leukocytes, kidney) and serum. It was considered possible that the stunted growth was related to development of antibodies against components of connective tissue. The present report deals with demonstration of fixed antibodies in various tissues of the sensitized animals.

Methods and materials. Preparation of extracts from rabbit tendon has been previously described(1). Since the previous study showed connective tissue extracts (C.T.) from old rabbits to be more antigenic than those from young animals, only tissue extracts from rabbits older than one year were used. Extracts from leg and foot tendons of guinea pigs were prepared in the same fashion and used as test antigen only. Guinea pigs of the Hartley Strain weighing approximately 300 g received weekly injections of C.T. suspended in Freund's adjuvant. The injection aliquots contained 150 μg of connective tissue protein

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* The authors are grateful to Lemuel Hall for technical assistance and to Medical Illustration Service for preparing the photographs.

TABLE I. Weight Gain of Guinea Pigs during First 35 Days* of Experiment.

	No. of animals	Initial wt range (g)	Wt gain (g)	
			Range	Mean & S.D.
A. Inj. with C.T. in adjuvant	30	238-330	96-273	195 ± 47
B. ” ” adjuvant and saline	5	264-332	243-302	273 ± 22
C. Uninjected	7	266-320	180-310	245 ± 44
D. B and C combined	12	264-332	180-310	257 ± 37

Statistical comparison of wt gains of Groups B and C: t, 1.33; $p > 0.2$.
A and D: t, 4.04; $p < 0.001$.

* This is an arbitrarily chosen interval. The difference remained significant after longer intervals, but sample size diminished because of periodic killing of animals.

as determined with the technic of Lowry *et al.* (2).[†] After 4 such injections, monthly booster injections of half the dose were given. Guinea pigs were divided into 3 groups (Table I). Animals of group A were injected with C.T. in adjuvant. One group of control animals received injections of adjuvant and saline (group B), the other received no injections (group C). The guinea pigs were weighed each week. Twenty-three guinea pigs of group A and 8 control animals were bled by cardiac puncture on the 96th day of the sensitization regimen and their sera tested for C.F. antibodies to extracts of rabbit and guinea pig connective tissue (Table II). These animals were not sacrificed to permit long term observation. Nine guinea pigs of Group A and 7 guinea pigs of the control group were killed by exsanguination through the jugular vein in intervals from 13 to 68 days following start of injection regimen (Table II). Blood was collected and serum obtained in the usual manner for serologic, electrophoretic and hematologic studies. The animals were immediately dissected and tissue samples from the various organs were frozen in liquid nitrogen and sectioned in a cryostat. The sections, which were cut at a thickness of 5 μ, were placed on slides to which they adhered following thawing without addition of adhesive material. They were washed with buffered saline (pH 7.3-7.4) for three 10 minute periods, dried at room temperature, then overlaid with commercial fluorescein conjugated rabbit anti-guinea pig globulin(3). The latter had been previously mechanically agitated with powdered activated charcoal(4) at a concentration of 50 mg per ml of serum for one hour at room temperature and the charcoal removed at high speed centrifugation (18,000 rpm for 10 min.). Slides were kept overnight in a sealed moist chamber at 5°C, then washed with buffered saline (3x10 min.) and air dried. A drop of buffered glycerol (pH 7.4) was then added and cover slips applied. Microscopic examination was performed with fluorescent light illumination. A Zeiss fluorescent microscope was used with OSRAM HBO 200 light source, UG2 and

TABLE II. Guinea Pigs Sensitized with Rabbit Connective Tissue in Adjuvant.*

No. of animals	Day after start of sensitization	Reciprocal of titer of complement fixing antibodies to		Fluorescent antibodies
		Rabbit C.T.	Guinea pig C.T.	
2	13	neg.	neg.	neg.
		4	”	pos.
2	26	8	8	”
		8	neg.	”
1	34	8	”	”
1	40	neg.	”	”
1	47	4	”	”
1	60	32	4	”
1	68	128	8	”
23	96	32 (2) 64(10) 128 (7) 256 (4)	neg.(10) 4 (9) 8 (3) 32 (1)	n.d.†

* 16 animals served as control. Neither complement fixing antibodies nor fluorescence could be demonstrated in these animals.

† Not done.

[†] Other determined constituents of C.T. included hydroxyproline, uronic acid, hexose, hexosamine and sialic acid. We are grateful to Dr. L. Robert, Dept. of Biochemistry, Univ. of Illinois College of Medicine and Dr. R. D. Coleman, Senior Research Biochemist, VA West Side Hospital, for performing these studies which will be reported later in detail.

UG5 exciter filters, and BG23 and GG4 barrier filters. An attempt was also made to demonstrate fixation of anticonnective tissue antibodies to organ sections from normal

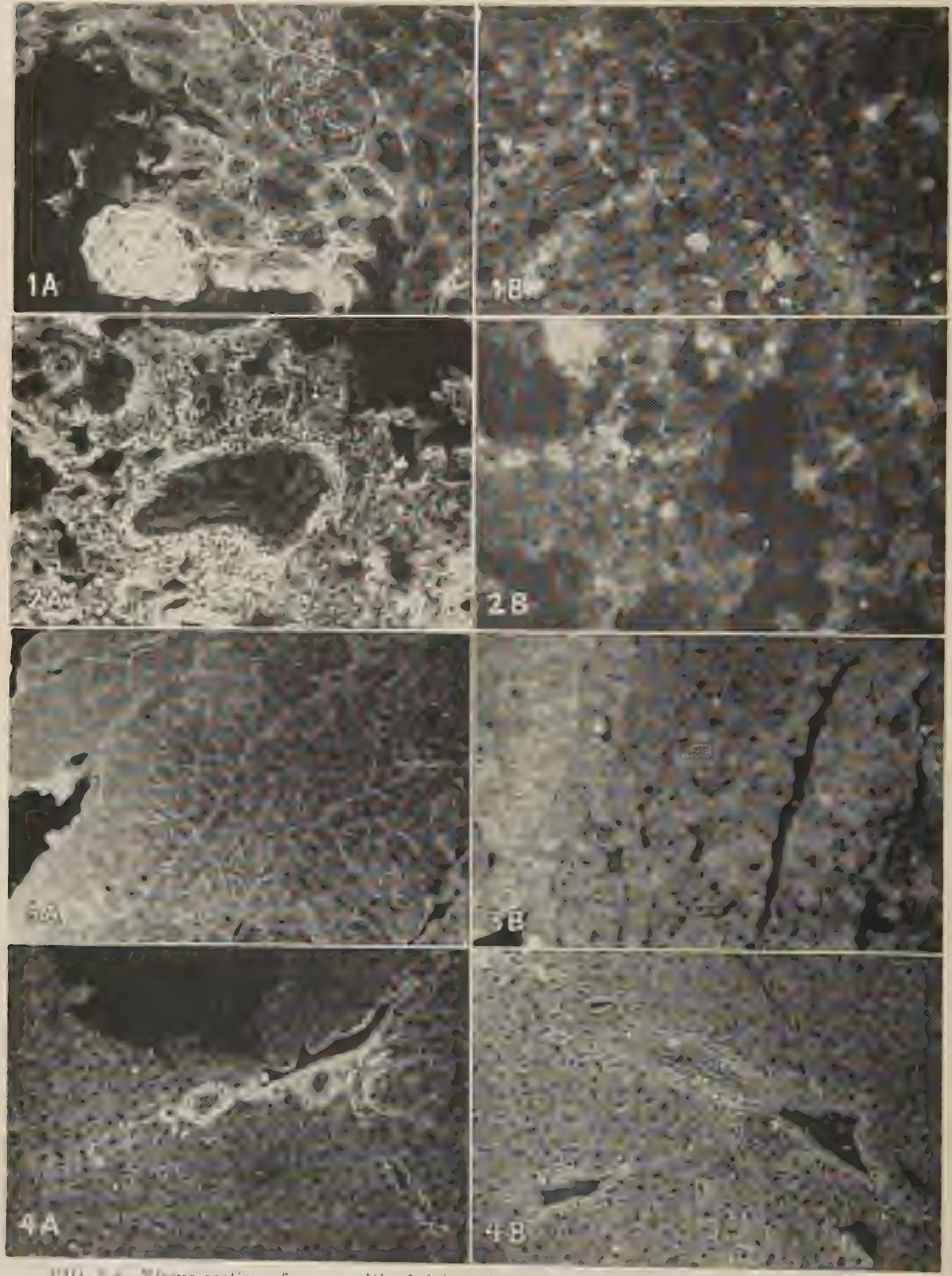

FIG. 1-4. Tissue sections from sensitized (a) and control (b) guinea pigs incubated with fluorescent antiserum. (1) Kidney, (2) Lung, (3) Heart, (4) Liver.

guinea pigs and rabbits. For this purpose these sections were overlaid with antiserum to rabbit C.T. and others with normal guinea pig serum. The slides were then placed in a moist sealed container which was mechanically rotated for one hour at room temperature. They were washed in buffered saline for 3 changes of 10 minutes each, air dried and overlaid with conjugated antiguinea pig globulin rabbit serum as described above. The test for C.F. antibodies was performed according to the standard technic of Kolmer.

Results. The weight gain of the group of animals injected with C.T. was markedly diminished as compared to control animals (Table I). In the examined tissue sections from 8 of the 9 guinea pigs injected with C.T., fluorescence was observed following incubation with the fluorescein conjugated antiguinea pig globulin (Fig. 1a-4a). The sections which did not show fluorescence came from an animal sacrificed 13 days following first injection. Fluorescence appeared to be localized in interstitial spaces, basal membranes and vascular walls of all examined organs, which included kidney (Fig. 1a), lung (Fig. 2a), heart (Fig. 3a), adrenals, spleen and liver (Fig. 4a). In kidney, fluorescence was most marked in the glomerular basement membrane; in lung, in the interalveolar septa and peribronchial tissue. In no organ was intracellular fluorescence demonstrated.

Tissue sections from normal guinea pigs overlaid with normal guinea pig serum followed by incubation with fluorescein conjugated antiserum did not reveal specific staining (Fig. 1b-4b). If, however, serum from guinea pigs which had been injected with C.T. were used, specific fluorescence was observed following incubation with the conjugated antiserum. As a control the same procedure was performed on tissue sections from 2 normal rabbits with similar results.

Complement fixing antibodies were observed in serum of 30 of 32 sensitized guinea pigs with rabbit C.T. as test antigen. Titers increased during sensitization and ranged from 1:4 to 1:256 (Table II). Sixteen guinea pigs also developed C.F. antibodies to guinea pig C.T. at titers of 1:4 to 1:32. Anti-connective tissue antibodies could not be demonstrated in control animals. In all animals injected with rabbit C.T., the electrophoretically determinable gammaglobulin fraction was higher than in non-injected animals. The Coombs test remained negative and the hematological examination did not reveal abnormalities.

In view of fixation of guinea pig globulin in tissue sections of parenchymatous organs of sensitized guinea pig, it was considered likely that serum of these animals would also react with saline extracts from such guinea pig organs in the complement fixation test. This was tested with tissue extracts from guinea pigs (liver, kidney, heart, lung). Titers were of same magnitude as titers of isologous antibodies to tendon extract.

Discussion. The presence of gammaglobulin in tissue sections as visualized by its specific reaction with a fluorescein labelled antigammaglobulin is generally interpreted to be the result of a specific antigen-antibody reaction in the tissue with fixation of the antibody to the antigenic tissue component. Our findings, therefore, appear to indicate fixation of anti-connective tissue antibodies in interstitial spaces and basement membranes and vascular walls of several organs. Since rabbit tissue was used for sensitization of guinea pigs, the presence of circulating antibodies in guinea pigs to the rabbit connective tissue is not surprising; however, induction of tissue fixed antibodies in guinea pigs appears to be consistent with the possibility that autologous antibodies had been induced by heterologous antigen. This appears to apply also to circulating C.F. antibodies although the reactions were not as strong as with the heterologous antigen (Table II). The reaction of guinea pig tissue with anti-rabbit tissue antibodies could also be demonstrated *in vitro* following incubation of tissue sections from organs of normal uninjected guinea pigs with anti-rabbit connective tissue serum. Sera of sensitized guinea pigs also reacted with isologous organ extracts. These phenomena suggest the possibility that the occasionally reported cross reactions(5,6,7) of antisera to various organs with other organs are caused by the antigenicity of ubiquitous connective tissue.

Goodman(8) has recently reported that rabbits injected with human liver nucleoprotein developed antibodies not only to human nucleoprotein extracts, but also to such extracts obtained from rabbit liver. The results of the present study suggest that auto- and iso-antibodies to connective tissue may also be induced by foreign material. It is uncertain if the stunted growth of the guinea pigs is related to development of these anti-connective tissue antibodies.

Identification of the antigenic component in connective tissue is under study.

Summary. Tissue fixed globulin has been demonstrated in interstitial tissue and basement membranes of various guinea pig organs following inoculation of guinea pigs with saline extracts of rabbit tendon. These animals had a significantly diminished growth. It is likely that the fixed globulin represents "auto-antibody" induced by heterologous antigen.

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Received June 2, 1960. P.S.E.B.M., 1960, v104.

Effect of Thyroxine and Propylthiouracil on Magnesium Metabolism in the Rabbit. Study with Mg^{28}.* (25918)

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The factors regulating the metabolism of magnesium are obscure. Previous studies have shown that insulin and glucose(1), cortisone(2), pyridoxine and desoxypyridoxine (3), and alloxan (unpublished) may affect distribution of Mg^{28} in rabbits. Very little is known about the relationship between magnesium metabolism and the thyroid gland, although it has been suggested that either hyperthyroidism or hypothyroidism may alter the partition of magnesium in serum(4). The present study was designed to investigate the effects of experimentally induced hyper- and hypo-thyroid states on magnesium metabolism in the rabbit. Before and after administration of thyroxine or propylthiouracil to 2 groups of animals, the external balance of magnesium was determined and exchangeable magnesium content was measured with Mg^{28}. Mg^{28} was also used to determine relative tissue radioactivity in rabbits which had been on larger doses of thyroxine or propylthiouracil.

Material and methods. Thirty-two normal domestic adult male rabbits were kept in individual stainless steel metabolism cages and were fed a stock diet of compressed pellets. Except in exp. 2 and 4, tap water was given without restriction. *Thyroxine sodium* was dissolved in physiologic saline solution made slightly alkaline with a few drops of 1 N NaOH, and was made up to a final concentration of 0.3 mg/ml. In preliminary observations, 7 rabbits were given 2 intravenous injections of thyroxine, 0.3 mg per kg of body weight, within 48 hours. A striking loss of body weight ensued, and 3 of the 7 rabbits died within 7 days. In the definitive experiment, therefore, only one dose of 0.3 mg/kg was administered initially. When it appeared

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* This work supported by contract between U. S. Atomic Energy Comm. and Univ. of Colorado.

that maximal sublethal effects were not developing, a second dose of thyroxine was administered. *Propylthiouracil,* U.S.P., was dissolved in distilled water and made up to a concentration of either 0.05% or 0.10%. *Serum and urine magnesium* determinations were performed by modification of molybdivanadate method for phosphate(5). *Radioactivity assay.* Samples of plasma and tissues were assayed for gamma ray activity with scintillation counter. A total of 10,000 counts were made on each sample, and all determinations corrected for physical decay of the isotope. *Exp. 1 and 2. External balance studies* for magnesium were performed for 15 days in 8 rabbits in *Exp. 1* and for 27 days in 8 rabbits in *Exp. 2.* Each morning before rabbits were fed, body weight, total food consumption, and 24-hour urine volume were measured. Each gram of feed was known to contain 0.1723 meq of magnesium. *Measurement of exchangeable magnesium content* (Mg_e). Mg_e determinations were made at weekly intervals in both groups. Method of preparing magnesium solution for injection has been described(6). Five ml of magnesium solution, containing/ml approximately 2 μcuries of Mg^{28} in 0.4 meq of untagged magnesium, was injected intravenously. Urine collected 20 hours after injection was pooled; between 24th and the 27th hour, 2 spot specimens were collected by catheterization, and their specific activities determined. The exchangeable magnesium content was calculated as previously described(7). *Exp. 1.* Control data were obtained during first 2 days. On second day, after completion of initial Mg_e determination, each animal received, by intravenous injection, 0.3 mg of thyroxine sodium/kg body weight. On eleventh day of experiment, 9 days after first dose of thyroxine sodium, each animal was given a second intravenous injection containing the same dose of thyroxine sodium. The third Mg_e determination was made on fourth day following this second dose of thyroxine (15th day of experiment). *Exp. 2.* Control data were obtained during first 4 days. Beginning on morning of fifth day, drinking water was replaced by 0.05% solution of propylthiouracil in distilled water. Each animal was given 250 ml of this solution to drink daily for 27 days. *Exp. 3 and 4. Tissue uptake studies. Exp. 3.* Sixteen rabbits were divided into 2 groups of 8 animals each. Each of 8 rabbits in test group was given 3 intravenous injections of thyroxine sodium (1.0 mg/kg) within 4 days. Mg^{28} was injected 24 hours after third dose of thyroxine, and animals were killed exactly 4 hours later. Tissues were then analyzed for radioactivity, as previously described(6). 8 animals in control group were not given thyroxine sodium, but were otherwise treated identically with those in the test group. *Exp. 4.* Six of surviving animals in Exp. 2 were given propylthiouracil in drinking water in concentration of 0.1% from days 28 through 32. On 32nd day, each animal received intravenous injection of Mg^{28}, and was killed 4 hours later for tissue analyses. The control group was the same as in Exp. 3. *Statistical methods. Exp. 1 and 2.* Each animal served as its own control. After differences between baseline values and values obtained at subsequent Mg_e determinations had been calculated for each animal, mean differences for the whole group were obtained. Significance of mean difference was determined by use of "t" test, a "P" of less than 0.01 being considered significant(8). *Exp. 2 and 4.* Mean relative activity of each type of tissue was determined for both test and control groups, and significance of differences between group means was then tested(7).

Results. Effects of Thyroxine. No significant changes were noted in body weight, food intake, urinary magnesium excretion, external magnesium balance, and Mg_e at 7 and 4 days after injection of first and second doses of thyroxine sodium respectively. A significant decrease in serum magnesium concentration, from 2.11 to 1.65 meq/l, was noted on second day after second injection of thyroxine.

In animals killed after receiving 3 mg of thyroxine/kg of body weight within 4 days, a significant increase was found in relative radioactivity of skin, liver, appendix, and heart (Table I). Muscle and bone showed no significant change when compared with respective mean values in control group.

TABLE I. Effects of Thyroxine and Propylthiouracil on the Relative Radioactivity of Tissues in Rabbits.

	Relative radioactivity*		
Tissue	Control group‡	Thyroxine group§	Propylthiouracil group‖
Muscle	.50 ± .10¶	.68 ± .05	.41 ± .63
Skin	1.25 ± .14	2.13 ± .26†	1.39 ± .15
Appendix	3.90 ± .23	7.15 ± .45†	5.70 ± .71
Lung	4.25 ± .26	3.98 ± .18	3.58 ± .17
Liver	5.10 ± .42	9.92 ± .82†	3.47 ± .21†
Heart	5.90 ± .99	10.00 ± .58†	7.89 ± .51
Kidney	11.00 ± .60	11.20 ± .78	10.24 ± .54
Bone	12.90 ±1.13	12.89 ± .91	6.24 ± .81†

* Relative radioactivity $= \dfrac{\text{cpm/g tissue}}{\text{cpm/ml plasma}}$ at time of death.

† Statistically significant difference when compared with control group.

‡ Mean values in 8 rabbits not treated with thyroxine or propylthiouracil.

§ Mean values in 8 rabbits receiving thyroxine sodium, 3 doses of 1 mg/kg in 4 days.

‖ Mean values in 6 rabbits receiving propylthiouracil (.05% solution for 4 wk, followed by .10% solution for 4 days).

¶ Stand. error.

Effects of propylthiouracil. One of the 8 animals given propylthiouracil died during second week, and another during third week. These 2 animals ate poorly, lost weight, and had terminal Mg_e values of 3.7 and 11.0 meq. In remaining 6 rabbits, body weight was significantly increased on 13th, 20th, and 27th days of propylthiouracil administration (Table II). Mean daily food intake was decreased during first week, and the urinary excretion of magnesium was decreased during second week. Mg_e was significantly decreased at end of third week. Initial mean value for protein-bound iodine was 4.1 μg/100 ml; at end of fourth week, it was 2.5 μg/100 ml.

When the rabbits were killed after 4 days on double dose of propylthiouracil, a significant decrease was found in relative radioactivity of liver and bone. The other tissues showed no significant changes.

Comments. Although administration of thyroxine in Exp. 1 produced clinical evidences of a hypermetabolic state—tachycardia, hyperpnea, and increased irritability—the external balance study revealed no significant changes in magnesium metabolism. Two days following second injection of thyroxine, serum magnesium concentration was significantly depressed, although exchangeable body content of magnesium was not significantly altered. In Exp. 3 tissue uptake of Mg^{28} was increased in heart, appendix, skin, and liver, but not in muscle and bone. The decrease in serum magnesium concentration may have been caused by redistribution of extracellular magnesium into the intracellular compartment of tissues showing increased radioactivity.

Rabbits fed propylthiouracil were less active than controls, and values for protein-bound iodine, suggest that they were indeed hypothyroid. Tissue uptake of Mg^{28} by liver and bone was suppressed, and Mg_e was significantly depressed.

Previous studies demonstrated increase in exchangeable body content of magnesium under circumstances which produced an increase in relative radioactivity of *muscle* or *bone*. For instance, administration of cortisone to rabbits caused an increase in relative radio-

TABLE II. Effects of Propylthiouracil on Magnesium Metabolism in Rabbits.†

	Mean baseline values	Mean values at			
		Wk I‡	Wk II§	Wk III‖	Wk IV¶
Wt (kg)	2.519	2.483	2.670*	2.769*	2.795*
Serum Mg (meq/l)	1.72	1.79	1.57	1.86	1.79
Exchangeable Mg (meq)	73.6	54.5	51.4	42.3 *	77.5
Mg intake (meq/day)	23.1	19.6 *	20.7	20.2	20.3
Urinary Mg excretion (meq/day)	6.9	4.8	4.6 *	6.5	7.9
Urine volume (ml/day)	155	72 *	80 *	87 *	98 *

* Statistically significant difference when compared with mean baseline value.

† Propylthiouracil administered as 0.05% solution in distilled water, 250 ml daily.

‡ Mean values in 8 rabbits, 5 days on propylthiouracil.

§ " " " 7 " , 13 " " " .

‖ " " " 6 " , 20 " " " .

¶ " " " 6 " , 27 " " " .

activity of muscle, accompanied by increase in Mg_e. In contrast, the change in Mg^{28} uptake following administration of desoxypyridoxine, pyridoxine, or alloxan occurred in tissues other than muscle or bone, and was not associated with any significant alteration in Mg_e. In the present studies, although relative radioactivity in some soft tissues was increased by thyroxine, muscle and bone were not affected and no change occurred in Mg_e. Conversely, administration of propylthiouracil suppressed uptake of Mg^{28} in bone, and was associated with a significant decrease in Mg_e.

Previous studies showed that the largest body pool of magnesium is in muscle and bone. Magnesium in other tissues appears to exchange fairly rapidly with tracer dose of magnesium labeled with Mg^{28} and injected intravenously(6). In normal human subjects Mg^{28} does not enter muscle, bone, or erythrocytes in appreciable amounts, and Mg_e values are extremely low(7).

The results of our study suggest that, although thyroxine or propylthiouracil produces alterations in magnesium metabolism, which can be demonstrated by Mg^{28}, the changes are insufficient to be detected by usual external balance technic. In *potassium,* another cation which is predominantly intracellular in location, a decrease in *extra*cellular concentration reflects a depletion of the *intra*cellular store. Such a simple relationship does not seem to hold true with magnesium. Even though administration of propylthiouracil suppressed bone uptake of Mg^{28} sufficiently to decrease the exchangeable body content of magnesium, serum magnesium concentration did not change.

Summary. 1) In rabbits given thyroxine or propylthiouracil, external balance studies for magnesium were performed, and Mg^{28} used to measure exchangeable body content of magnesium. In 8 rabbits given 2 intravenous injections of thyroxine sodium (0.3 mg/kg) no significant changes were noted in body weight, magnesium balance, or exchangeable magnesium content, although a decrease in serum magnesium concentration occurred. In another group of 8 rabbits the tissue relative activity of Mg^{28} was determined following 3 intravenous injections of thyroxine (1.0 mg/kg) given within 4 days. The relative activity was increased in liver, skin, appendix, and heart. 2) Eight rabbits given propylthiouracil orally showed a decrease in exchangeable body content of magnesium at end of third week. A significant decrease was found also in relative radioactivity of liver and bone. 3) The value for exchangeable body content of magnesium appears related to rate of turnover of Mg^{28} in bone and muscle, the 2 largest body stores of magnesium.

Mg^{28} was supplied by Brookhaven Nat. Lab. on allocation from U. S. Atomic Energy Comm.

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Received June 6, 1960. P.S.E.B.M., 1960, v104.

Rat Anterior Pituitary Nucleic Acid Levels as Function of Age.[*] (25919)

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During a study of rat anterior pituitary gland nucleic acid levels following adrenalectomy(1), the content of ribonucleic acid (RNA) for control animals varied from 8-12 μg/mg of tissue. Since animals were not all of the same age, this variability might have been due to pooling of data obtained from rats of different ages. Secondly, the manner by which animals were sacrificed might be a factor influencing nucleic acid levels if RNA content is subject to change with stress. Therefore, this investigation was designed to study RNA content of anterior pituitary tissue of rats of specified age groups, sacrificed by either decapitation or an overdose of ether. In addition, water and collagen contents of the tissue were measured to note their levels as a function of age since fluctuations in these 2 constituents can influence RNA concentration.

Methods. Male rats of Holtzman strain were obtained at various ages: Group I—35-37 days old, Group II—51-59 days old, Group III—70-72 days old, Group IV—93-94 days old, Group V—128-132 days old, and Group VI—one year old. They were maintained on Purina Laboratory Chow pellets and tap water *ad lib.* in temperature-controlled animal quarters. Animals were sacrificed by decapitation or overdose of ether, pituitary glands removed, posterior lobe tissue discarded, and anterior lobes weighed on torsion balance. Tissue collected from each rat was homogenized in cold normal saline to make a 0.6% homogenate. Determination of RNA content from pituitary homogenates followed procedure described by Schneider(2). Water content of pituitaries from each age group was determined by weighing anterior lobe tissue before and after heating to dryness in oven at 100°C until a constant minimum weight was obtained. A chemical analysis for hydroxyproline was performed[†] on dried pituitary tissue as an estimate of collagen content(3).

TABLE I. Anterior Pituitary RNA Content of Various Age Groups Killed by Decapitation or by Overdose of Ether.

	Ant. pituitary RNA content (μg/mg tissue)	
Age group	Decapitation	Ether
35- 37 days old		7.1 ± .2*(38)
51- 59	6.4 ± .2*(24)	8.8‡± .4 (24)
70- 72	8.5†± .6 (10)	7.9 ± .4 (9)
93- 94	6.5 ± .2 (10)	6.8 ± .4 (6)
128-132	6.7 ± .3 (16)	7.1 ± .1 (6)
1 yr old		7.4 ± .6 (17)

* Stand. error. No. of rats in parentheses.

† Difference from decapitated groups II, IV and V statistically significant ("t" test for significance).

‡ Difference from ether groups I, IV and V and decapitated group II statistically significant.

Results. Data obtained for RNA content of anterior pituitary tissue of rats of various ages are summarized in Table I. For the most part, the concentration of RNA in anterior lobe of pituitary gland appears fairly constant.

In the series utilizing decapitation as means of sacrifice, there are no significant differences between Groups II, IV, and V. The figure obtained for rats in Group III killed by decapitation is, however, significantly higher than the remainder of the series.

Among the animals sacrificed by an overdose of ether, only those in Group II demonstrate a significant difference from most other groups. Likewise, it is only this group that shows any difference in RNA content dependent upon manner of sacrifice. Decapitated rats of Group II had 6.4 ± 0.2 μg of RNA/mg of tissue whereas the same age group subjected to ether overdosage had 8.8 ± 0.4 μg of RNA.

The water content of pituitary tissue from different age groups varied within a narrow range. Samples of tissue lost from 68-76% of wet weights following drying. Likewise, amount of collagen in tissue, calculated from hydroxyproline values, did not vary significantly for various age groups. Amount of collagen in tissue samples of all age groups studied was between 0.5 and 1.5%.

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[*] This investigation supported by research grant from Nat. Inst. of Arthritis, P.H.S., and by Arthritis and Rheumatism Fn.

[†] The hydroxyproline determinations were performed by Dr. T. B. Rosenthal of this department.

Discussion. The content of RNA in anterior pituitary tissue does not vary markedly from one age group to another. Higher values obtained for rats of Group III sacrificed by decapitation cannot be explained by any alteration in experimental procedure. For most age groups studied, there are no significant differences in RNA values obtained after sacrificing rats by either of the 2 methods. Stress produced by subjecting animals to ether is probably of too short a duration to influence nucleic acid changes. No explanation can be offered to account for the higher RNA values observed in Group II rats sacrificed by ether overdosage compared to the same age group sacrificed by decapitation.

Tissue content of RNA is not influenced by variation of degree of hydration or collagenous connective tissue infiltration from one age group to another. There are no differences in these 2 constituents among various age groups. Use of hydroxyproline determinations as index of amount of collagen present is reliable. Only collagenous connective tissue contains this amino acid in any appreciable amount(4).

Summary. A study was made of RNA, water, and collagen contents of adenohypophyses of rats of various age groups from 35 days to one year old, sacrificed by decapitation or an overdose of ether. There were no significant differences in level of RNA in tissue from one age group to another. Concentration of anterior pituitary nucleic acid was not influenced by manner of sacrifice for most age groups studied. Contents of water and collagen in the tissue were similar in young and old rats.

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Received June 6, 1960. P.S.E.B.M., 1960, v104.

Dihydrotachysterol (AT 10) and Mammogenesis in Ovary-Thyro-Parathyroidectomized Rats.* (25920)

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Continuing study of mammogenesis in ovary-thyro-parathyroidectomized female rats, influence of estradiol benzoate (EB) and progesterone (P) with thyroxine (T4) and dihydrotachysterol (AT 10) were compared.

Materials and methods. Sprague-Dawley-Rolfsmeyer rats were ovary-thyro-parathyroidectomized, injected, killed, and DNA of mammary glands determined under standardized conditions described(1). AT 10‡ was given every second day by stomach tube. Completeness of thyroidectomy checked by I^{131} uptake described(1). Group I received 1 μg EB plus 3 mg P. Group II received in addition 20 μg/100 g AT 10. Group III re-

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[*] Contribution from Mo. Agr. Exp. Sta. Journal Series No. 2167. Approved by Director.

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[‡] Kindly supplied by Sterling-Winthrop Research Inst., Rensselaer, N. Y. The commercial solution of AT 10 or dihydrotachysterol (Hytakerol) contains about 1250 μg of active principle per ml.

TABLE I. Effect of Dihydrotachysterol (AT 10) on Mammogenesis in Ovary-Thyro-Parathyroidectomized Albino Rats.

Daily treatment from day 15-33	No. of rats	Body wt			Pituitary wet wt/100 g BW (mg)	48 hr uptake of I^{131}		6 posterior mammary glands		
									DNA	
		Day 1 (g)	Day 34 (g)	Gain (%)		Neck (%)	Body (%)	DFFT, total/100 g BW (mg)	µg/mg DFFT (µg)	Total/100 g BW (mg)
EB: 1 µg; P: 3 mg	14	244	277	14 ± 2.1	$6.6 \pm .13^{1}$	1.2 ± .10	$3.3 \pm .32^{10}$	227 ± 10^{19}	$35 \pm .8^{28}$	$7.9 \pm .22^{37}$
Idem + AT 10: 20 µg/100 g	14	226	254	12 ± 1.5	$7.6 \pm .22^{2}$	2.3 ± .27	$4.9 \pm .54^{11}$	276 ± 6^{20}	$34 \pm .6^{29}$	$9.3 \pm .17^{38}$
Idem + T4: 1.5 µg/100 g	13	237	261	10 ± 1.3	$6.0 \pm .14^{3}$	.4 ± .05	$1.2 \pm .17^{12}$	227 ± 7^{21}	$34 \pm .7^{30}$	$7.7 \pm .23^{39}$
EB: 2 µg; P: 6 mg*	37	228	257	13 ± .8	$7.7 \pm .20^{4}$	1.1 ± .12	$1.8 \pm .20^{13}$	229 ± 7^{22}	$36 \pm .9^{31}$	$8.1 \pm .21^{40}$
Idem + T4: 3 µg/100 g	33	228	254	11 ± .81	$6.2 \pm .18^{5}$	.2 ± .03	$.4 \pm .04^{14}$	204 ± 5^{23}	41 ± 1.0^{32}	$8.3 \pm .19^{41}$
EB: 2 µg; P: 6 mg; AT 10: 10 µg/100 g	16	235	261	11 ± 1.3	$7.9 \pm .27^{6}$	1.0 ± .09	$3.0 \pm .25^{15}$	238 ± 8^{24}	$33 \pm .8^{33}$	$7.9 \pm .23^{42}$
Idem + T4: 3 µg/100 g	15	226	255	13 ± 2.1	$6.7 \pm .24^{7}$	.3 ± .05	$.8 \pm .10^{16}$	209 ± 6^{25}	$35 \pm .5^{34}$	$7.4 \pm .16^{43}$
EB: 2 µg; P: 6 mg; AT 10: 20 µg/100 g	20	224	247	10 ± 1.7	$8.5 \pm .35^{8}$	2.0 ± .22	$4.6 \pm .58^{17}$	268 ± 7^{26}	$32 \pm .6^{35}$	$8.5 \pm .22^{44}$
Idem + T4: 3 µg/100 g	16	247	267	8 ± 1.9	$6.9 \pm .23^{9}$	.4 ± .08	$1.1 \pm .13^{18}$	230 ± 6^{27}	$36 \pm .7^{36}$	$8.1 \pm .20^{45}$

Student's "t" probability

Groups	p
1–2 2–3 4–5 8–9 11–12 13–14, 17 14–16, 18 15–16 17–18	$<.001$
19–20 20–21 22–26 26–27 31–32 32–34, 36 35–36 37–38 38–39	$<.001$
6–7 13–15 38–40	$.001 < p < .005$
4–7 22–28 24–26 31–35	$.005 < p < .01$
4–9 5–9 10–11 15–17 24–25 25–27 38–44	$.01 < p < .025$
4–8	$.025 < p < .05$
5–7	$.05 < p < .1$
22–25 31–33 40–44 42–43 44–45	$.1 < p < .2$
40–41	$.2 < p < .4$
22–24	$.4 < p < .5$

* Data from (1).

BW = body wt
DFFT = dry, fat-free tissue
EB = estradiol benzoate
P = progesterone
T4 = thyroxine
AT 10 = dihydrotachysterol (Hytakerol)

ceived in addition 1.5 μg T4/100 g. Group IV received 2 μg EB plus 6 mg P and 10 μg AT 10/100 g. Group V received in addition 3 μg T4/100 g. Group VI received 2 μg EB, 6 mg P plus 20 μg AT 10/100 g. Group VIII received in addition 3 μg T4/100 g.

Results. In OTPEC rats 1 μg EB plus 3 mg P without either T4 or parathyroid hormone replacement therapy stimulated mammary gland growth (as measured by DNA) equal to that stimulated in OE animals (Table I)(2). When 20 μg AT 10/100 g was added, DNA was increased significantly to 9.3 mg/100 g due to a significant increase in DFFT. Addition of 1.5 μg/100 g T4 depressed favorable effect of AT 10 resulting in DFFT and DNA comparable to effect of EB and P alone.

Same significant increase of DFFT was observed when 20 μg AT 10 was added to 2 μg EB plus 6 mg P. However, increase in total DNA did not occur due to a significant reduction in DNA/mg DFFT. Addition of 3 μg/100 g T4 again reduced DFFT but DNA/mg DFFT was increased resulting in total DNA comparable to EB and P alone.

Total DNA of groups receiving 10 μg AT 10/100 g alone and with 3 μg T4/100 g were not significantly influenced in comparison to 2 μg EB plus 6 mg P, although again T4 depressed DFFT slightly.

Wet weight of pituitaries of EB plus P injected rats are increased significantly in comparison to normal controls(1) but were further significantly increased with 20 μg AT 10/100 g. Addition of T4 reduced this hyperplasia markedly.

Completeness of thyroidectomy was proven by I^{131} uptake at 48 hours on day 33 or 34. Intact control animals showed 9.87 ± .84% in neck region and .91 ± .05% in body, whereas operated animals showed highly significant lower uptake in neck region (Table I). All animals treated with AT 10, with or without T4, showed significantly increased I^{131} uptake in comparison with other groups.

Discussion. Data presented confirm previous observation that normal mammogenesis can be induced experimentally with adequate levels of ovarian hormones in OTPEC rats (1). Effectiveness was not changed when 1 μg EB plus 3 mg P instead of 2 μg EB plus 6 mg P were given. 20 μg AT 10/100 g/day, a level far below "toxic borderline dose" of 100 μg AT 10/100 g/day(4) exerted significant positive effect on mammogenesis.

In previous study(5) T4 was shown to cause a significant increase in total DNA when 3 μg and 6 μg/100 g were injected with 2 μg EB plus 6 mg P in OE rats. DFFT was maintained and an increase in DNA/mg DFFT was observed.

In normal pregnant animals T4 at levels of 2.5 and 3.5 μg/100 g increased total DNA by maintenance of DFFT and increase of DNA/mg DFFT (unpublished).

In present study T4 added to AT 10 at either 1.5 or 3 μg/100 g level depressed total DNA by significantly depressing DFFT without producing a corresponding increase of DNA/mg DFFT observed in ovariectomized and pregnant animals. It is suggested that if parathyroid hormone acts similar to AT 10 in stimulating total DNA and further that T4 increases parathyroid hormone secretion rate, then the results obtained with T4 in ovariectomized and pregnant animals could be harmonized with present observations.

Favorable effect of T4 in non-TPEC animals might be due, in part, to increased secretion of parathormone. Higher levels of AT 10 may show increased total DNA of mammary glands when administered with T4.

Increased uptake of I^{131} in OTPEC rats administered AT 10 raises question of its role in I^{131} uptake, release and T4 secretion rate in normal animals.

Summary. Daily administration of 1 μg EB plus 3 mg P to mature ovary-thyro-parathyroidectomized rats for 19 days stimulated mammary gland growth (DNA) comparable to ovariectomized rats receiving same treatment. Replacement therapy with 10 and 20 μg AT 10/100 g/day, respectively, in combination with 1 μg EB plus 3 mg P increased total DNA significantly. No benefit was observed by increasing ovarian hormones to 2 μg EB plus 6 mg P. Combined administration of AT 10 at above mentioned levels and 1.5 μg and 3 μg T4/100 g depressed DFFT significantly resulting in reduced total DNA.

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Received June 6, 1960. P.S.E.B.M., 1960, v104.

Production of Incomplete Vi Antibody in Mice. (25921)

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Landy(1) reported that Swiss Bagg mice respond to isolated Vi antigen by producing specific antibody demonstrable by the standard hemagglutination test(2), and that the passive protection afforded by this antibody was sufficient to account for the immunogenic activity of the antigen. These findings have been confirmed for BALB/c mice (an inbred variant of the Bagg), the strain employed most extensively in our laboratory. Recently, however, it was observed that specific antibody could not be demonstrated in the serum of Vi-immunized Cinnamon mice, a strain not previously employed by us for serological studies. Inasmuch as Cinnamon, as well as BALB/c mice, could be protected actively against challenge with virulent *Salmonella typhosa* by administration of Vi antigen, this observation was unexpected, and suggested that response to antigenic stimulation might differ with the strain of mouse employed. The present study was therefore designed to elucidate and compare responses of these 2 strains of mice to Vi antigen. Evidence was obtained that the type of antibody evoked by Vi antigen does indeed vary with the mouse strain. The antibody developed in Cinnamon mice immunized with Vi antigen was of the incomplete type, but was nonetheless protective against challenge with *S. typhosa*.

Materials and methods. Mice. The animals ranged in weight from 11-16 g, and were of mixed sex. BALB/c mice were obtained from Microbiological Associates, Inc., Bethesda, Md., and Cinnamon mice from Dept. of Lab. Animal Medicine, Walter Reed Army Inst. of Research. *Vi Antigen.* The purified Vi antigen was prepared from the Vi strain of *Escherichia coli* (5396/38) by the method of Webster *et al.*(3). Immediately prior to use, antigen was dissolved in 0.85% NaCl to give desired concentration. All inoculations were made intraperitoneally. *Sera.* Specimens for use in passive tests and for serological study were prepared as follows: mice were inoculated with 10 μg of Vi antigen, bled 6 days later, and the serums pooled. *Absorption of sera.* 0.3 ml of packed bacterial cells (killed by heating at 60°C for 1 hr) was added to each ml of serum, the suspension incubated at 37°C for 1 hr, at 4°C overnight, and clarified by high speed centrifugation. *Passive protection tests.* Tests for serum potency were based on use of graded immunizing doses and a constant challenge dose, and were performed in Cinnamon mice. Serum was given intraperitoneally 1 hour prior to challenge with a saline suspension of 3 x 10^7 *S. typhosa* Ty2V (fully virulent form) *via* the same route. ED_{50}s were determined by the method of Reed and Muench(4). *Serological procedures.* The hemagglutination test for complete (saline) Vi antibody was performed as described by Landy and Lamb(2). The procedure for demonstration of incomplete Vi antibody was the same except that serum dilutions were made in 15% bovine serum albumin (BSA) obtained from Armour Pharmaceutical Co.

Results. During our studies on the role of Vi antigen in virulence of *S. typhosa* for mice,

TABLE I. Response of Cinnamon and BALB/c Mice to Purified Vi Antigen.

Vi antigen (μg)	Vi hemagglutinin titers (saline)*	
	Cinnamon serum†	BALB/c serum†
10	Neg‡	1:240
1	”	1:120
.1	”	1:60
.01	”	1:7.5
.001	”	Neg

* Serum diluted in .85% saline.

† Pooled from 20 mice bled 6 days following a single inj. of Vi antigen.

‡ Neg = < 1:7.5.

it was observed that Cinnamon mice failed to produce demonstrable Vi antibody in response to administration of this antigen, in contrast to the response observed in BALB/c mice. These findings are shown in Table I. As little as 0.01 μg of purified Vi antigen elicited production of complete (saline) Vi antibody in BALB/c mice, while as much as 10 μg of antigen failed to stimulate production of this antibody by Cinnamon mice. As indicated previously, both Cinnamon and BALB/c mice could be actively immunized against challenge with virulent *S. typhosa*. Based on quantity of Vi antigen required to protect half the animals, the extent of protection afforded the 2 mouse strains was essentially the same. Employing a challenge of 3.36 x 10^7 Ty2V cells, the ED$_{50}$s of Vi for Cinnamon and BALB/c mice were 0.0078 and 0.0098 μg respectively. In the case of the BALB/c animals, the amount of Vi antibody produced was sufficient to account for the immunogenic activity of this antigen, but there was no demonstrable antibody to account for the protection afforded Cinnamon mice following administration of Vi antigen. Consequently, the serum of Vi-inoculated Cinnamon mice was examined for its capacity to protect passively. Groups of mice were inoculated intraperitoneally with graded doses of pooled serum from Cinnamon mice previously immunized with Vi antigen; 1 hour later, they were challenged with virulent typhoid bacilli (strain Ty2). For control purposes, other groups of mice were passively immunized with pooled "immune" BALB/c serum and similarly challenged. Results of a typical experiment (Table II), show that serum obtained from Vi-immunized Cinnamon mice gave protection to the same extent as that afforded by serum from Vi-immunized BALB/c mice.

In the absence of demonstrable Vi antibody, the protective capacity of serum from Cinnamon mice suggested the presence of an atypical antibody, which might still account for the observed protection. The findings (Table III) clearly show that although saline antibody is absent (Table I), an incomplete type of Vi antibody is present in the serum of Vi-immunized Cinnamon mice. It is noteworthy that the titer of serum from Vi-inoculated BALB/c mice diluted in albumin was invariably higher than the saline titer.

TABLE II. Passive Protection Provided by Sera from Vi-immunized Cinnamon and BALB/c Mice.

Mouse serum*	Serum dose (ml) .05	.01	.002	ED$_{50}$ (ml)†
Cinnamon	9/10‡	6/10	2/10	.007
BALB/c	10/10	7/10	”	.005

* Pooled from 20 mice bled 6 days following a single inj. of 10 μg of Vi antigen.

† Challenge: 3 × 10^7 *S. typhosa* Ty2V.

‡ Survivors/No. inj.

From the findings given in Tables II and III, it is apparent that incomplete antibody accounted for the protection afforded mice by immunization with Vi antigen. To ascertain the specificity of this protection, serum of immunized Cinnamon mice was subjected to specific absorption procedures to remove its antibody and protective capacity. Serum from Vi-immunized BALB/c mice was treated in the same way, and these absorbed sera were then examined to determine whether they were still protective and serologically reactive. Table IV reveals that absorption of both Cinnamon and BALB/c sera with a Vi-containing organism markedly reduced

TABLE III. Production of Incomplete Vi Antibody by Cinnamon and BALB/c Mice.

Vi antigen (μg)	Vi hemagglutinin titers (BSA)*	
	Cinnamon serum	BALB/c serum
10	1:240	1:1920
1	1:60	1:960
.1	1:15	1:480
.01	Neg	1:120
.001	”	1:7.5

* Serum diluted in 15% bovine serum albumin (BSA).

TABLE IV. Specificity of Protection Afforded by Cinnamon and BALB/c Sera. Effect of absorption.

	Cinnamon serum*				BALB/c serum*			
	Dose (ml)				Dose (ml)			
Treatment of serum	.05	.01	.002	ED_{50} (ml)†	.05	.01	.002	ED_{50} (ml)†
Unabsorbed	9/10‡	6/10	2/10	.007	10/10	7/10	2/10	.005
E. coli (Vi-containing)	2/10	0/10	0/10	>.05	2/10	1/10	1/10	>.05
S. typhosa Ty2W (non-Vi variant)	9/11				10/10			

* Pooled from 20 mice bled 6 days following a single inj. of 10 μg of Vi antigen.

† Challenge: 3 × 10^7 *S. typhosa* Ty2V.

‡ Survivors/No. inj.

Challenge controls: 10 mice were inj. with normal Cinnamon serum, 10 with normal BALB/c serum, and 10 with saline prior to challenge. There were no survivors.

protective capacity of both sera. On the other hand, absorption with a W form (non Vi-containing) *S. typhosa* culture was without effect on the protection afforded by these sera. Similarly, both incomplete and complete Vi antibody were absorbed from these sera by treatment with Vi-containing bacilli, but not by absorption with non-Vi cells (Table V). The findings that protective activity and antibody content of these sera could be removed by absorption with Vi-containing organisms provide evidence for the specific nature of the responses of both Cinnamon and BALB/c mice to administration of Vi antigen.

Discussion. Incomplete antibodies have been detected in experimental animals and in human beings, but much of the investigative work on this type of antibody has been along serological lines. Relatively little is known of the protective capacity of sera containing only incomplete antibody, despite observations that sera which apparently contain immune antibodies as shown by protection tests, fail to show serological activity (agglutinins or precipitins). The presence of incomplete antibody in serologically non-reactive sera has been suggested by some workers(5) as a possible explanation for the passive protection provided by such sera. Landy(1) reported that serum of mice injected with a heat-phenol vaccine prepared from a Vi-containing strain of *S. typhosa* afforded a limited amount of protection to Bagg strain mice in absence of antibody detectable *in vitro*. Based on the results of absorption experiments, he attributed the protective activity of the serum to Vi antibody, and stated that such antibody differed in some way from the readily demonstrable Vi antibody produced in response to more potent immunizing agents such as acetone-killed and dried Vi-containing organisms or isolated Vi antigen. On the other hand, Tyler and Swingle(6), investigating the effect of photo-oxidation of anti-pneumococcal rabbit serum and anti-diphtheric horse serum concluded that "univalent" type antibody is ineffective for passive protection against bacteria, but may function as protective antibody against toxins. The present study, however, has shown that incomplete Vi antibody effectively protects Cinnamon mice against challenge with virulent typhoid bacilli.

The protection afforded by normal and certain so-called immune sera against a variety of virulent organisms, and enhancement of phagocytic activity by such sera in the absence of readily detectable antibody may also be due to specific incomplete antibody. Tests

TABLE V. Specificity of Vi Antibody in Cinnamon and BALB/c Sera. Effect of absorption.

	Cinnamon serum*		BALB/c serum*	
	Vi antibody titer†			
Absorbant	Saline	BSA	Saline	BSA
Unabsorbed	Neg	1:240	1:240	1:1920
E. coli (Vi-containing)	"	Neg	Neg	Neg
S. typhosa Ty2W (non-Vi variant)	"	1:240	1:240	1:1920

* Pooled from 20 mice bled 6 days following a single inj. of 10 μg of Vi antigen.

† Determined by Vi hemagglutination test. Serum dilutions made in 0.85% saline or 15% bovine serum albumin.

by diagnostic, clinical laboratories on sera of patients suffering from infectious diseases seldom, if ever, include determinations for incomplete antibody. If present, such antibody might provide a more useful measure of the progress of the infection. Potuzník and Gavlík(7) have published data comparing incomplete and complete Vi antibody in serum of typhoid patients and carriers, and found that incomplete Vi antibody appeared in higher titers in almost every case, particularly among the carriers.

It is generally believed that during the many years of its use, typhoid vaccine has afforded man a significant degree of protection against natural infection. The fact that Vi antibody, as determined by the usual procedures, appears in a relatively small percentage of immunized individuals has led a number of investigators to question the importance of Vi antibody in protection of man against this disease. In the light of the present work, however, it might be profitable to reexamine sera from typhoid-vaccinated individuals for incomplete Vi antibody. Such a study is currently in progress in this laboratory.

Summary. Immunization with purified Vi antigen elicited incomplete Vi antibody in the Cinnamon strain, and complete Vi antibody in BALB/c mice. Incomplete Vi antibody alone accounted for protection afforded by serum from Cinnamon mice against challenge with fully virulent typhoid bacilli. Absorption tests verified the specificity of incomplete Vi antibody and protection it afforded.

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Received June 6, 1960. P.S.E.B.M., 1960, v104.

Growth and Passage of Variola Virus in Mouse Brain.* (25922)

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Variola virus has been distinguished from other pox viruses by its poor growth on primary inoculation into laboratory animals, and its failure to become adapted to laboratory animals on subsequent passage(1,2,3). Furthermore, it has been reported that mice inoculated intracerebrally (IC) with variola virus succumb in erratic death patterns(4), and that lethality disappears after the first passage(3). Under proper conditions to be described here, it will be shown that these distinguishing characteristics may not serve as absolute markers for differentiating variola from the other pox viruses. Data are presented which show that variola virus does grow well when inoculated IC in mice, and that it can be serially passaged.

Materials and methods. The Yamada strain of variola virus was used. To determine whether multiplication of virus occurred, a large number of Swiss mice were inoculated IC. At selected sampling intervals, the brains of 3 sacrificed mice were pooled, homogenized as 20% suspensions in heart infusion broth, and assayed for virus titer on the chorio-allantoic membrane (CAM) of 11-day-old embryonated eggs(5). Ten eggs were used for each serial 10-fold dilution. Titers were expressed

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* We are indebted to Dr. A. W. Downie, University of Liverpool, England, for making a microfilm of Dr. Helbert's thesis available, and supplying additional information on variola infection in animals.

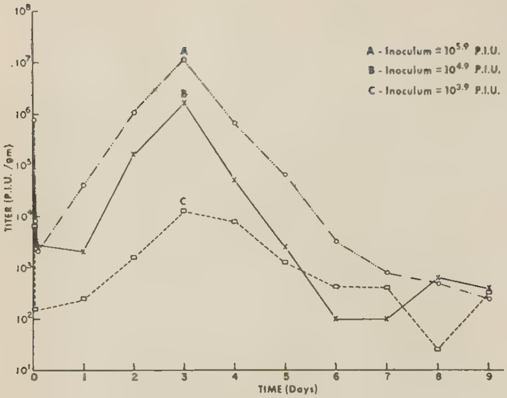

FIG. 1. Growth of variola virus in mouse brain.

as number of pock infectious units (p.i.u.)/g of tissue.

Results. The results of a typical experiment on growth of virus in the brain of IC inoculated mice weighing 12-14 g† are shown in Fig. 1. Titer of the virus decreased 91-99% in the first 5 minutes, rose to a peak between 48 and 72 hours, then decreased. In experiments using younger mice (8-10 g), this final rate of decrease was more gradual. The peak titer was generally proportional to inoculating dose and occurred on the same day irrespective of size of inoculum. These 2 features are not characteristic of most viruses. Generally, peak titer is independent of inoculating dose and is usually delayed with low doses. The growth curve obtained with the highest dose confirms Helbert's results(6) who was one of the first to give quantitative growth data in mice in a comparative study of variola and alastrim.

Preliminary experiments suggested that the marked initial decline in titer was neither due to dilution effect(7) nor to very rapid and efficient adsorption to susceptible cells. The first explanation was ruled out when almost complete recovery of unadapted Newcastle disease virus was obtained 5 minutes after IC inoculation. The second explanation was considered unlikely because of the well documented reports on slow and inefficient adsorption of pox viruses(8,9), and the reproducibly lower decrease in titer obtained with brain-adapted vaccinia virus in the same interval of 5 minutes (average 80%, vaccinia; average 95%, variola). The possibility for existence of a virus inhibitor in mouse brain is being investigated.

The virus was uniformly lethal for mice when $10^{5.5}$ p.i.u. or more were inoculated intracerebrally. With smaller doses, mortality rate and death pattern became erratic (See also(2) and (6). For example, although doses as low as 10 p.i.u. infected 50% of the mice inoculated, as determined by resistance to challenge with neurotropic vaccinia virus after 3 weeks, the percentage dying varied from one experiment to another. Furthermore, in any given experiment, percentage of dead mice from this very small dose was sometimes the same or greater than percentage dying from a larger dose.

Rough estimates indicated that weanlings (6-8 g) were approximately 1000 times more susceptible to the lethal effect of the virus than older (12-14 g) mice. Quantitative pock counts showed that the younger mice consistently produced 90% more virus in brain tissue. Marshall and Gerone (manuscript in preparation) have found that suckling mice 6-22 hours old give consistently uniform death patterns such that confident assays may be carried out in this host.

Serial passage of variola virus by the IC route was next attempted. The brains of three 8-10 g mice, each inoculated with $10^{5.5}$ p.i.u., were excised at time of peak titer (3rd day) and homogenized as a 20% suspension in heart infusion broth. Inoculation of these homogenates proved to be toxic for a certain proportion (10-30%) of the mice which succumbed within the first 15 minutes. The toxic factor was associated with uninfected mouse brain, but by repeated low speed centrifugation, the toxicity of the supernatants was substantially reduced. Passages were made, therefore, employing both supernatants and whole brain homogenates. Ten consecutive passages were made. The results of one experiment (Table I) indicated that variola virus may be continuously propagated by intracerebral passage provided the requirements of dosage and time of harvest are met. The erratic lethal pattern persisted when titrations

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† 12-14 g = 28-30 days; 8-10 g = 21-23 days.

TABLE I. Passage of Variola Virus Intracerebrally in Mice. 10 successive passages.

Series 1 Homogenized brain suspension passaged Titer p.i.u./g	Series 2 Supernatant of brain suspension passaged Titer p.i.u./g
1.2×10^7	
7.0×10^6	1.1×10^5
1.7×10^7	1.5×10^6
1.0×10^6	2.2 "
4.0 "	2.0×10^4
8.0×10^5	4.0×10^2
2.3×10^6	2.5 "
4.9×10^7	1.5 "
3.5 "	6.0 "
1.6 "	8.0 "

of passage material were made. It is noteworthy in the passages made with supernatants, that once a decrease in titer occurred (5th passage), the titer remained low and could not be restored. This occurred twice in 6 passage series. In both cases, when the titer fell below a critical level of about 10^4 p.i.u./g, it decreased further on passage and remained at a very low level.

Attempts were made to confirm the identity of the tenth passage virus and to determine if a variant of variola virus might have emerged. The results of these experiments (Table II) identified the passaged virus as variola virus. Mice surviving infection with the passaged virus were immune 3 weeks later to IC inoculation of 3000 MIC LD_{50}s of a mouse neurotropic strain of vaccinia virus. Pocks that formed on the CAM by the tenth passaged material were the same in size, color and morphology as those formed by the parent variola strain. The passaged virus, like the parent, was incapable of forming plaques on monolayers of chick fibroblasts. Pirsch and Mika(10) have found that with the exception of variola and alastrim, all the classical pox viruses tested form distinct plaques on chick fibroblast monolayers. This appears to be an important new characteristic differentiating variola from other members of the pox group. These and the remaining data in Table II support the fact that a variant had not emerged during the 10 mouse brain passages. Passaged mouse brain material and an egg seed prepared from it gave identical results.

Summary. Variola virus was lethal for mice by intracerebral route when relatively high doses were inoculated. Lower doses produced erratic death patterns. Virus proliferated in mouse brain reaching peak titer between 48 and 72 hours. Amount of virus at peak titer was dependent on quantity of virus inoculated. Ten consecutive passages of the virus in mouse brain were successfully carried out. The most important factor which appeared to influence successful serial passage of the virus was level of titer attained in preceding passage. This titer was, in turn, dependent on size of inoculating dose, time of harvest and age of mouse. The passaged virus was identical with parent strain with respect to a

TABLE II. Properties of Parent and Mouse Brain-Passaged Variola Virus.

Property	Parent virus	Passaged virus
Surviving mice challenged with neurotropic vaccinia virus (3000 MIC LD_{50}s)	Induces immunity	Induces immunity
Pocks on CAM	White convex .9 mm 2 days 1.4 3	White convex .9 mm 2 days 1.4 3
Plaques on chick fibroblasts	None	None
Lethality for mice	Erratic death pattern (see text)	Erratic death pattern
Growth response in mouse brain	Same as Fig. 1	Same as Fig. 1
Ratio $\text{p.i.u./g} \,/\, \text{r.s.l.p.}_{50}\text{/g}^*$	$10^{2.5}$	$10^{2.5}$
Hemagglutinin titer	1/160	1/160
50°C inactivation rate (first 30 min.)	1.7 log/15 min.	1.6 log/15 min.

* p.i.u./g = pock infectious units/g of CAM seed; r.s.l.p._{50}/g = median rabbit skin lesion-producing dose/g of CAM seed.

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Received June 7, 1960. P.S.E.B.M., 1960, v104.

A New Insight into Pathogenesis of Carbon Tetrachloride Fat Infiltration.* (25923)

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Recent investigations in a number of laboratories have made possible the formulation of a simple hypothesis regarding the underlying functional derangement responsible for accumulation of fat in the liver following carbon tetrachloride poisoning. This hypothesis is as follows: The liver is constantly secreting large quantities of triglycerides into the plasma. Carbon tetrachloride poisons the secretory mechanism, and as a result triglycerides accumulate in the liver. Key observations which led to formulation and testing of this hypothesis were the following: It was observed(1) that following intubation of carbon tetrachloride into the stomach of rats, the hydrocarbon reached its peak concentration in the liver within 1 to 2 hours. In another study(2) it was shown that liver triglycerides were elevated 34% within one hour and 195% within 3 hours following carbon tetrachloride poisoning. Furthermore, we have shown that mitochondrial oxidation of octanoate, and mitochondrial respiratory control, are completely unaffected 2 hours after carbon tetrachloride poisoning at which time liver triglycerides are elevated 70% (Recknagel, R. O., and B. Lombardi, unpublished). These observations in conjunction with our earlier studies(3,4) of mitochondrial function following carbon tetrachloride poisoning, as well as related work of others(5) appeared to us to demonstrate convincingly that failure of fat oxidation, or uncoupling of oxidative phosphorylation, could not account for the early rise in liver triglyceride content. It was further observed(6) that 4 hours after carbon tetrachloride poisoning, hepatic glucose-6-phosphatase, a microsomal enzyme, was depressed to 59% of control levels. Subsequently, (Recknagel, R. O. and B. Lombardi, unpublished) it was shown that this enzyme was depressed 30% 2 hours after carbon tetrachloride intoxication. Neubert and Maibauer (7) had previously noted that the hepatic microsomal enzyme system involved in aminopyrine detoxication was destroyed in carbon tetrachloride poisoned rats at a time when no derangement in mitochondrial oxidative phosphorylation was evident. These observa-

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* Supported by grants from Nat. Inst. of Arthritis and Metab. Dis. and Nat. Heart Inst., N.I.H., P.H.S.

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tions pointed to the membranous component of the endoplasmic reticulum as the most probable locus for a hitherto unknown mechanism intimately concerned with hepatic lipid metabolism. Consideration of the role of liver in transport of lipids in the body led to the hypothesis that an hepatic lipid secreting mechanism was probably involved. The recent reviews by Fredrickson and Gordon(8) and by Olson(9) may be consulted for the historical roots of this hypothesis. More recently, Byers and Friedman(10), by preventing exit of triglycerides from the plasma by means of Triton injection, demonstrated convincingly that the liver is capable of secreting large amounts of triglycerides into the plasma. It appeared to us to be highly likely that this hepatic triglyceride secretory mechanism was the locus of a functional derangement in the carbon tetrachloride poisoned liver. This communication presents data in support of this hypothesis.

Methods. Sixteen male rats of the Sprague-Dawley strain, from 208 to 274 g body weight, were used. The rats were without food overnight for 17 hours. The experiment which was carried out twice was designed for 8 rats divided into 4 equal groups. Four rats were force-fed mineral oil (0.25 ml/100 g body weight), and 4 rats were force-fed a 1:1 carbon tetrachloride-mineral oil mixture (0.5 ml of the mixture/100 g body weight). Force-feeding was done through a stomach tube under light ether anesthesia. After 2 hours, 2 of the mineral oil fed rats were injected with 0.9% NaCl and 2 were injected with Triton (Winthrop Labs, WR-1339). Similarly, carbon tetrachloride fed rats were injected with either 0.9% NaCl or Triton. The dose of Triton was 100 mg/rat, dissolved in 0.5 ml of 0.9% NaCl, injected into a saphenous vein under light ether anesthesia. Ninety minutes after Triton (or 0.9% NaCl) injection, 5 ml of blood were withdrawn from the abdominal aorta. The liver was removed and weighed, and a liver sample taken for triglyceride analysis. Blood and liver samples were taken under sodium pentobarbital anesthesia (5 mg/100 g body weight, intraperitoneally). Plasma triglycerides were estimated according to Van Handel and Zilversmit(11). Total liver lipid was extracted according to Folch *et al.*(12), redissolved in chloroform and passed over a silicic acid column. The chloroform eluate of the silicic acid column was analyzed for triglycerides according to Van Handel and Zilversmit(11).

TABLE I. Effect of Carbon Tetrachloride Poisoning on Plasma Triglyceride Content in Triton Treated and Non-Triton Treated Rats.

Treatment of animals	No. of rats	Plasma triglycerides, mg/100 ml plasma	Liver triglycerides, mg†
Control	4	24.4 ± 2.65	20.5 ± 2.93
CCl_4 fed	4	6.4 ± .86	60.9 ± 8.84
Triton (no CCl_4)	4	286 ±21.6	19.3 ± 3.68
CCl_4 fed + Triton	3*	69 ±10.3	63.0 ± 4.33

* One rat lost under nembutal anesthesia.

† All data given as mean ± stand. error. Liver triglycerides given as mg/whole liver/100 g body wt.

Results. Ninety minutes after Triton administration to normal rats, plasma triglyceride content was elevated 12-fold (Table I). If the rats had been poisoned with carbon-tetrachloride 2 hours previously, this increase in plasma triglycerides was markedly depressed. Plasma triglyceride concentrations in carbon tetrachloride poisoned rats not given Triton were also markedly lower than corresponding controls. Data of Table I also indicate that 3.5 hours post carbon tetrachloride feeding, liver triglyceride content has been elevated 3-fold. Triton administration did not alter hepatic triglyceride concentration in normal rats, nor did it affect hepatic triglyceride accumulation due to carbon tetrachloride. This suggests that insofar as lipid transport mechanisms are concerned, Triton selectively inhibits exit of triglycerides from plasma whereas carbon tetrachloride selectively inhibits exit of triglycerides from liver. Plasma triglyceride values formed 4 distinct groups with no overlapping values. Liver triglyceride values formed 2 distinct groups (CCl_4-fed *vs.* non-CCl_4-fed) with no overlapping values.

Discussion. The data presented suggest that with respect to the hepatic triglyceride secretory mechanism, carbon tetrachloride has, in effect, produced a functional hepatectomy. This is evident in the non-Tritonized animals, but the effect is markedly exaggerated following Triton administration. Failure of the triglyceride secretory mechanism appears to be almost complete 2 hours after intoxication, which corresponds in time with attainment of the peak concentration of carbon tetrachloride in the liver(1). Failure of the hepatic secretory mechanism results in a dramatic fall in plasma triglycerides and a corresponding retention of triglycerides within the liver. The small rise of plasma triglycerides in the carbon tetrachloride poisoned rats injected with Triton could be due in part to a contribution from the gut(10).

The hypothesis in its present form has been restricted to the pathology of carbon tetrachloride poisoning. It has been tested only in regard to the action of this hepatotoxin. However, it appears self-evident to us that the hepatic triglyceride secretory mechanism must be complex and that it could fail as a result of a variety of pathological disturbances of the liver cells. It will therefore be most interesting to determine whether ethionine administration, choline deficiency, phosphorus poisoning, etc. likewise produce hepatic fat accumulation by destruction of or interference with the normal function of the hepatic triglyceride secretory mechanism. The elegant demonstration of this hepatic function by Byers and Friedman(10) has provided a simple method for determining the extent to which the hypothesis here presented is in fact a general one and not peculiar to carbon tetrachloride poisoning alone.

Summary. Intravenous administration of Triton to rats causes a marked elevation of plasma triglycerides. If the rats have been poisoned with carbon tetrachloride 2 hours previously, accumulation of triglycerides in the plasma following Triton administration is markedly reduced. The existence of an hepatic triglyceride secretory mechanism is inferred from the work of others, and it is postulated that carbon tetrachloride poisons this triglyceride secretory mechanism. Triglycerides, which would normally be secreted into plasma, are retained in the liver.

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Received June 8, 1960. P.S.E.B.M., 1960, v104.

Adrenal Ascorbic Acid and Corticosteroidogenesis Following Unilateral Adrenalectomy in the Rat.* (25924)

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In spite of widespread use of adrenal ascorbic acid concentration as parameter of adrenal cortical activity, little is known regarding its true relationship to corticosteroidogenesis. Since a sensitive method for deter-

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* This investigation supported by research grant (B-959) from Inst. of Neurol. Diseases and Blindness, N.I.H.

mination of corticosterone is now available, it appeared of interest to correlate changes in ascorbic acid and in adrenal and plasma corticosterone concentration, observed as ordinates of time following unilateral adrenalectomy in the rat.

Materials and methods. One hundred and seventeen adult male Sprague-Dawley rats, distributed into one group of controls and 16 of experimentals, were acclimatized for 7-10 days to laboratory conditions (constant lighting and temperature (25 ± 1°C); Purina Fox-Chow and water *ad lib.*) in batches of 6-12. In view of extreme sensitivity of corticosteroidogenic response to environmental change(1), they were caged singly and left undisturbed for 12 hours prior to experiment. Left adrenalectomy, used as stimulus and as means of securing an internal control for remaining gland, was performed by lumbar approach, under ether anesthesia. To prevent stress-induced elevation of its corticosterone concentration,[†] the control gland was removed within 90 seconds of anesthesia. The animals were killed by decapitation at progressively longer intervals[‡] (1.5, 2, 4, 5, 10, 15 and 30 minutes; 1, 2, 4, 10, 24, 48, 72, 96 and 192 hours) from onset of etherization. Arteriovenous blood was collected from neck vessels into heparinized beakers for plasma corticosterone determination. Right adrenals of experimentals and both glands of controls were dissected free of adhering tissue and halved. The halves were weighed to nearest 0.05 mg and respectively extracted, following homogenization with rod and sand, in ethanol-saline for corticosterone, and 4% trichloroacetic acid for ascorbic acid determination. Adrenal and plasma corticosterone was determined by modification(2) of Silber's fluorometric technic(3); adrenal ascorbic acid, by modification(4) of Roe and Kuether's method (5). Results were expressed as percent changes from left adrenal levels for adrenal corticosterone and ascorbic acid, and from control baseline for plasma corticosterone.

TABLE I. Resting Levels of Adrenal Ascorbic Acid and Corticosterone in Control and Experimental Groups.

Group	Gland	Ascorbic acid, γ/100 mg fresh tissue	Corticosterone, γ/g fresh tissue
Controls (17)*	Left	462.2 ± 9.2†	8.4 ± 1.3
	Right	467.7 ± 13.6	10.1 ± 1.3
Exp. (100)	Left	462.8 ± 7.5	8.6 ± .5

* No. of animals. † Stand. error.

Results. Resting levels. No significant difference was observed (Table I) between left and right adrenals of controls and left glands of experimentals, with regard to ascorbic acid and corticosterone concentrations. With due correction for residual fluorescence (6), mean plasma corticosterone level in controls was 3.3 ± 0.8 γ/100 ml.

Postoperative levels. A fall of adrenal ascorbic acid (Fig. 1A), detected within 4-5 minutes of surgical procedure and maximal (50%) after 1-2 hours, was followed by a

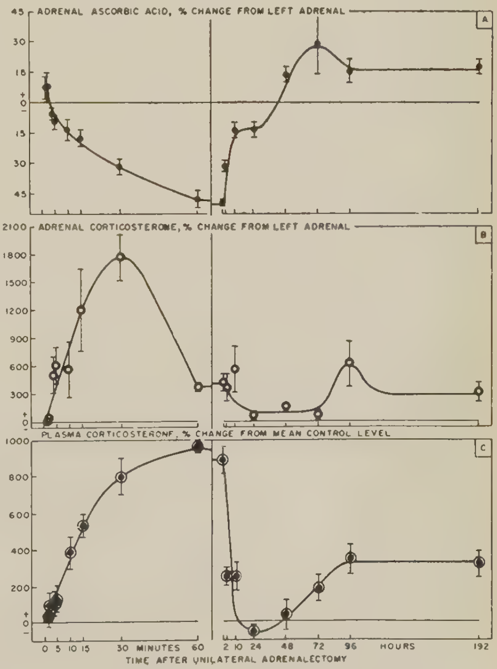

FIG. 1. Effects of unilateral adrenalectomy on ascorbic acid (A) and corticosterone (B) concentrations of remaining gland, and on plasma corticosterone level (C). Stand. errors are indicated by T-shaped bars.

[†] In few instances when left adrenal corticosterone concentration exceeded 20 γ/g, inadvertent stimulation was suspected and the data discarded.

[‡] Antibiotics were administered on first postoperative day to animals maintained 48 hours or more after unilateral adrenalectomy.

rise interrupted at 10th hour, resumed at 24th, and levelling off above baseline after 3-4 days. Adrenal corticosterone response to the stimulus (Fig. 1B) was characterized by a 17-fold increase in concentration within 30 minutes, followed by return to baseline after 24 hours, and secondary rise after 3 days to moderately elevated values recorded on 4th and 8th day. The pattern evidenced by plasma corticosterone (Fig. 1C) differed by lower peak (ninefold increase) maintained one additional hour, and followed by regression to distinctly subnormal (40%) level after one day. Concentration increased, thereafter, to plateau of 300% observed between 4th and 8th day.

Discussion. The immediate postoperative changes in recorded parameters of adrenocortical activity are admittedly related to the release of ACTH triggered by surgical procedure. Hodges and Vernikos(7) showed that, under comparable conditions, level of ACTH in blood reaches its peak within 2½ minutes and is back to normal after 20. Since peak adrenal and plasma corticosterone values, and maximal ascorbic acid depletion were not recorded before 30 to 60 minutes postoperatively, it appears that the responses initiated by ACTH outlive its disappearance from blood. This persistent activation, noted by Brodish and Long(8) with respect to adrenal ascorbic acid, is ascribed by them to ACTH adsorbed by the gland and which, according to Sonenberg *et al.*(9), presents an exponential rate of disappearance. Duration and rate of corticosteroidogenesis could be likewise conditioned by amount of adsorbed ACTH and its biological half-life in the adrenal. This is consistent with the observed long-lasting effect of preincubation with ACTH on *in vitro* steroid production of rat adrenals subsequently washed and incubated in an ACTH-free medium(10).

No significance should be attached to the relatively smaller percentile increase of plasma than of adrenal corticosterone values, since baseline for plasma was obtained from intact donors, and should therefore be halved for strict comparison with values from unilaterally adrenalectomized animals.

The rise and fall of corticosterone in the gland are respectively indicative of a predominance of synthesis over release of hormone during first 30 minutes, and of release over synthesis from then on. It is interesting that ascorbic acid depletion appears more closely related in time to release than to synthesis of corticosterone, since maximal depletion does not correspond to peak of corticosterone concentration in the gland (30 minutes), but is achieved at the same time (60-120 minutes) as the peak of its concentration in plasma, when rate of release of the hormone presumably reaches equilibrium with rate of removal or inactivation. As a corollary, ascorbic acid repletion and fall of plasma corticosterone are initiated simultaneously, 2 hours postoperatively. This temporal relationship between initial changes in ascorbic acid concentration and release of corticosterone should, however, be interpreted with caution, in view of recorded instances of dissociation between the 2 phenomena(11,12). The relationship was not apparent, beyond the first postoperative day, when a secondary rise of plasma corticosterone paralleled continued repletion of adrenal ascorbic acid. Moreover, since the observed rise of plasma corticosterone concentration preceded by 2 days a corresponding change of its adrenal concentration, it would be difficult to dissociate the possible involvements, in early part of rise, of slower rate of inactivation and of increased rate of release of the hormone. The eventual rise of adrenal corticosterone concentration between third and fourth postoperative day is tentatively ascribed to the effect of transient hypocorticoidism recorded after 24 hours, on feedback-mediated release of ACTH.

Summary. From comparative study of changes in adrenal ascorbic acid and in adrenal and plasma corticosterone concentration induced by unilateral adrenalectomy in the rat, adrenal ascorbic acid depletion appears more closely related in time to release than to synthesis of corticosterone, since maximal depletion did not correspond to peak of corticosterone concentration in the gland (30 minutes postoperatively), but was achieved at the same time (60-120 minutes p.o.) as its

peak concentration in plasma, when rate of release of the hormone presumably reached equilibrium with its rate of removal or inactivation. In view of reported instances of dissociation between the 2 phenomena, the observed temporal relationship should be interpreted with caution.

The authors are indebted to Miss R. C. Wood for able technical assistance.

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Received June 9, 1960. P.S.E.B.M., 1960, v104.

Actual Length of Diastasis for Regurgitation through Normal Mitral Valve; Cinefluorographic Demonstration. (25925)

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Regurgitation of blood can occur through a normal mitral valve. This has been demonstrated by: Hayden, *et al.*(1) utilizing isotope dilution; Friedman *et al.*(2) recording blood impedance changes after injecting sodium chloride; Wiggers(3) and Little(4), in measuring changes in pressure curves; and by Daley, *et al.*(5), using Evans blue in hearts with auricular fibrillation. Paul(6) has recorded this phenomenon by cinefluorography using opaque contrast in dogs with a slowed heart rate. The present study was undertaken as a further elaboration of Paul's work to determine the minimum length of diastasis to permit minimal regurgitation.

Method. Mongrel dogs, weighing 6.8-20.4 kg were anesthetized with sodium pentobarbital (35 mg/kg, *i.v.*). A No. 6 French whistle-tipped catheter was introduced *via* a thoracotomy incision into the left atrium through the auricular appendage and brought out through a stab wound in the chest. Pericardium and chest wall were closed, lung was fully expanded, and spontaneous respiration permitted to reappear. Two catheters (Nos. 6 F and 10 F) were then introduced into the left ventricle *via* the 2 common carotid arteries. The No. 6 F catheters in the left atrium and ventricle were attached to previously balanced strain gauges and pressures recorded simultaneously on a multichannel recorder. These pressures were subsequently used to measure period of diastasis (Fig. 1). After the heart was slowed by stimulation of the left vagus (tied off proximally) contrast material was rapidly injected into the left ventricle *via* the No. 10 F catheter. The opaque medium was visualized by means of a Philips fluoroscopic image intensifier(7,8) and

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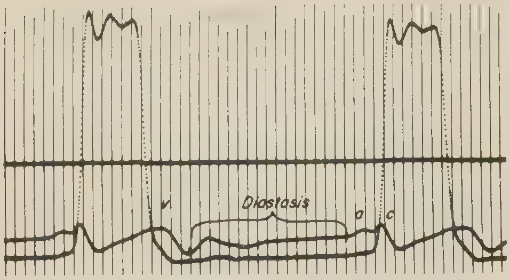

FIG. 1. Pressures from left ventricle and left atrium were recorded simultaneously to measure duration of diastasis. Time interval equals 0.04 sec.

recorded on 16 mm film with a Bell and Howell camera at 64 frames per sec. All dogs were in the supine position and a horizontal X-ray beam was used. Contrast material utilized was 60, 70, or 90% sodium methylglucamine diatrizoate, 70% sodium diprotrizoate or 70% sodium acetrizoate. The dose varied from 1 to 2 ml/kg. Location of the mitral valve was determined by its known relationship to the fat pad in the left atrio-ventricular sulcus and to the opacified left circumflex coronary artery. Contrast material posterior to these structures indicated presence of mitral regurgitation.

Results. A total of 270 injections were made in 18 dogs and diastolic regurgitation was observed in all animals in which there was sufficient prolongation of diastasis (Fig. 2). There is a critical duration of diastasis for regurgitation to occur. No regurgitation occurred when duration of diastasis was shorter than this period, but was always present when diastasis was longer than this critical interval (Table I). Amount of regurgitation increased directly with prolongation of this diastolic period. The average length of diastasis for minimal regurgitation was found to be 0.39 sec. (Table II). This value varied from 0.22-0.60 sec. but a fairly constant time was found for each animal.

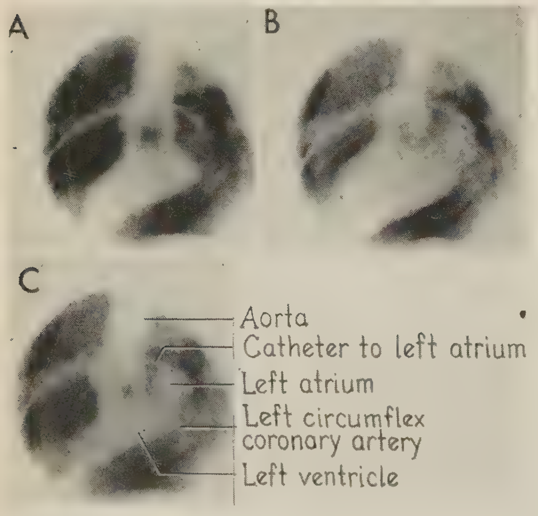

FIG. 2. Selected frames from cinefluorographic study show diastolic regurgitation with slowed heart rate: (A) control, no regurgitation; (B) mitral regurgitation with prolonged diastasis due to slowed heart; (C) subsequent ventricular systole.

TABLE I. Examples of Successive Recordings during the Production of Minimal Diastolic Regurgitation.

Dog #18		Dog #12	
Diastasis (sec.)	Regurgitation	Diastasis (sec.)	Regurgitation
.01	None	.01	None
.44	"	.16	"
.18	"	.32	"
.44	1+	.78	2+
.66	"	.70	1+
.42	None	.68	"
.50	1+	.60	"
.70	"	.48	"
.52	None	.38	None
.50	"	.40	1+
		.38	None
		.38	1+
		.32	None
		.34	"
		.28	"
		.88	2+

1+ = Barely perceptible with motion.
2+ = Definite regurgitation in single frame.

When the No. 10 F catheter was coiled in the left ventricle, or pushed as far as possible against the apex, or deliberately placed through the mitral valve into the left atrium no regurgitation appeared until heart rate was slowed. Similar results were obtained in 10 different dogs after both catheters were removed from the left ventricle and injection was made directly through a needle in the left ventricle.

Systolic regurgitation was seen in 4 out of 18 dogs. This occurred throughout the study and was independent of heart rate. With a slow heart rate and consequent prolongation of the diastasis, diastolic regurgitation also occurred. These dogs were subsequently

TABLE II. Total Series in Production of Minimal Regurgitation through Normal Mitral Valve by Prolonging Diastasis in 11 Dogs.

No. of inj.	Length of diastasis in sec. at which min regurgitation appeared	No. of inj.	Length of diastasis in sec. at which min regurgitation appeared
14	.34	*22	.22
*21	.46	18	.38
19	.28	18	.32
19	.48	17	.32
9	.60	15	.44
16	.42		
		Avg	.39

* Had minimal regurgitation with diastasis of 0.1 sec. with several inj., but could not be reproduced.

found at autopsy to have hemorrhagic mitral valves.

Discussion. The mechanism of mitral regurgitation through a normal valve has been discussed(6,9,10). This phenomenon of regurgitation can readily be recorded with the cinefluorographic technic by slowing heart rate and thus prolonging diastasis beyond a critical point.

Actual determination of this critical time interval was subject to certain limitations. Vagal stimulation often produced an irregular slowing of the heart, making it difficult to reproduce the same rate. Injections of contrast material were made by hand. These varied in speed and force, and were performed during different phases of the cardiac cycle. These factors resulted in considerable variation of amount of contrast material within the left ventricle at any one time. One dog had an irregular auricular rhythm making measurement of the periods of diastasis uncertain. There is a definite subjective element present in interpretation of "minimal" regurgitation.

Thus the average duration of diastasis for minimal regurgitation was found to be 0.39 sec., but because of the above noted limitations, considerable variations from 0.22-0.60 sec. were obtained.

Summary. Diastolic regurgitation of opaque contrast material can occur through normal mitral valve when the period of diastasis exceeds a specific time interval. This period of diastasis in dogs varies from 0.22 to 0.60 second, average of 0.39 second. When diastasis is shorter than this interval no regurgitation occurs, but it always occurs when prolonged beyond the critical period.

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Received May 9, 1960. P.S.E.B.M., 1960, v104.

Arteriosclerosis in the Rat: Aortic Enzyme Changes Accompanying Arterial Pathology.* (25926)

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Repeatedly bred rats have been shown to develop arteriosclerosis spontaneously(1-4). This tendency was greatly intensified by unilateral nephrectomy and injection of ACTH (2). This report describes changes in arterial enzyme concentrations which can be correlated with gross pathology.

Methods. Rats were 8- to 9-month-old female breeders from Sprague-Dawley Farms. The majority had 4 to 6 pregnancies, and averaged 330 g in body weight. Animals were used soon after receipt and no treatment was given prior to sacrifice. Animals were sacrificed by decapitation, and their aortas classified as follows: No grossly discernible lesions = *clear;* thin annular streaks in abdominal aorta = *minimal;* annular streaks plus plaques in the arch = *moderate;* plaques throughout the arch, and thoracic and abdominal aorta = *severe.* The entire aorta was removed and the lumen flushed with normal saline. The aorta was weighed after removal of excess moisture and adherent connective tissue, and a 5% homogenate prepared in ice-cold saline with hand operated Tenbroeck tissue grinder. Disintegration of tissue was complete in 3 to 5 minutes. The homogenates were centrifuged at 1000 times gravity for 10 minutes to prepare supernatants for lactic dehydrogenase (LDH) and glucose-6-phosphate dehydrogenase (G-6-PDH) assay. The 6-phosphogluconic dehydrogenase (6-PGDH) was assayed on supernatants obtained by centrifugation at 5000 times gravity for 10 minutes. Enzyme determinations were carried out by modification of Sobel procedure(5). Measurements were made in Beckman DU of rate of change in optical density at 340 mμ at one minute intervals. Assay systems contained TPN or DPNH and are described in legends of Tables I, II and III. Protein content of supernatants was determined by method of Gornall(6).

Results. Enzymatic activities (Tables I, II and III) were calculated from kinetic data obtained during periods when reaction rates were linear with time. Assays were terminated in 5 to 15 minutes. In control experiments, DPNH and TPNH were stable in solutions of buffered enzyme equivalent in concentration to those used in assays. In the absence of substrate, optical density at 340 mμ remained constant for periods exceeding the time required for assay.

Enzymatic activities are expressed as μ-moles of substrate reacting/unit time. Data in first column of Tables I, II and III are based on actual enzymatic activity contained in supernatant protein. Data in second column are calculated assuming that no activity was lost during centrifugation.† Lehninger (9) has pointed out that the aorta is relatively acellular and that extracellular connective tissue contributes little or no metabolic activity. We believe that soluble protein of aorta provides much more significant basis for calculation of enzymatic activity than wet weight. This method also tends to make measurements independent of mucopolysaccharide accumulation, collagen and fibrin deposition and calcification characteristic of pathologic aortas in these animals(4).

Part of TPNH, appearing during assay of G-6-PDH was due to presence of 6-PGDH in the extracts. Some of the 6-phosphogluconate formed in G-6-PDH reaction underwent further reaction with excess TPN to produce additional TPNH. Under these conditions ac-

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* This work supported by John A. Hartford Fn., Gustavus and Louise Pfeiffer Fn., Am. Heart Assn. and U.S.P.H.S.

† No significant differences in LDH, G-6-PDH and 6-PGDH could be detected when whole homogenates were compared to centrifuged preparations.

TABLE I. Changes in "Hexose Monophosphate Shunt"* Activity in Extracts of Aortas of Arteriosclerotic Female Sprague-Dawley Rats.

Gross pathology and No. animals	Enzyme activity		P (t test)
	μmoles TPNH produced/g soluble protein/min.	μmoles TPNH produced/g wet tissue/hr	
None (6)	42.9 ± 4.0†	133.8 ± 12.5	
Minimal "	38.6 ± 1.8	124.8 ± 5.8	N.S.‡
Moderate "	25.5 ± 3.1	91.8 ± 11.2	<.01
Severe "	32.0 ± 3.1	107.4 ± 10.4	<.05

Assay system: 0.1 M Tris buffer, pH 7.4; 1×10^{-2} M $MgCl_2$; 1.2×10^{-4} M TPN; 3.4×10^{-4} M glucose-6-phosphate; supernatant from 5% homogenate (.025 ml). Total vol 1 ml.

* The observed kinetics are due to combined action of G-6-PDH and 6-PGDH.

† Means ± stand. errors.

‡ Not significant.

tivity units in Table I express maximum capacity of the 2 linked enzyme systems to produce TPNH *via* "hexose monophosphate shunt." Data of Table III indicate that TPNH production by 6-PGDH was approximately 15 to 20% of the total. Data in Table I show that TPNH production by these 2 enzymes is significantly less in extracts obtained from aortas with *moderate* and *severe* pathology. LDH activity was also significantly decreased in these groups (Table II). No significant differences were observed in 6-PGDH activity of normal and arteriosclerotic aortas (Table III). Additional data would be required to establish a lack of correlation between level of 6-PGDH and presence of gross arteriosclerosis.

Discussion. Increasing gross arterial pathology in repeatedly bred female Sprague-Dawley rats can be correlated with decreases in concentration of LDH extracted from homogenates of arterial tissue. Kirk and coworkers reported differences in mean values for LDH activity in normal and arteriosclerotic human aortas. They observed that pathologic aortas were frequently low in LDH activity (7).

TABLE II. Changes in Lactic Dehydrogenase Activity in Extracts of Aortas of Arteriosclerotic Female Sprague-Dawley Rats.

Gross pathology and No. animals	Enzyme activity		P (t test)
	μmoles DPNH consumed/g soluble protein/min.	μmoles DPNH consumed/g wet tissue/hr	
None (7)	370 ± 22	1924 ± 114	
Minimal "	358 ± 36	1933 ± 194	N.S.
Moderate "	288 ± 22	1728 ± 132	<.03
Severe (5)	253 ± 16	1416 ± 90	<.005

Assay system: 0.1 M phosphate buffer, pH 7.8; 1.2×10^{-4} M DPNH; 4.0×10^{-3} M Na pyruvate; supernatant from 5% homogenate (.005-.01 ml). Total vol 1 ml.

TPNH production by combined action of G-6-PDH and 6-PGDH is significantly lower in preparations obtained from aortas showing *moderate* and *severe* pathology. Kirk reported similar observations using human arteriosclerotic aortas(8).

In vitro enzymatic reaction rates do not necessarily reflect rate limiting processes *in vivo*. Nevertheless, changes observed in pyridine nucleotide linked enzymes could conceivably affect critical rate limiting processes in the intact animal.

TABLE III. Comparison of 6-Phosphogluconic Dehydrogenase Activity in Extracts of Aortas of Normal and Arteriosclerotic Female Sprague-Dawley Rats.

Gross pathology	No. animals	Enzyme activity	
		μmoles TPNH produced/g soluble protein/min.	μmoles TPNH produced/g wet tissue/hr
None	10	7.81 ± .88	40.6 ± 4.57
Arteriosclerotic*	6	8.16 ± 1.49	45.7 ± 8.34

Assay system: 2.7×10^{-2} M glycylglycine buffer, pH 7.5; 1×10^{-2} M $MgCl_2$; 1.2×10^{-4} M TPN; 4.2×10^{-4} M Na 6-phosphogluconate; supernatant from homogenate (.2 ml). Total vol 1 ml.

* This group includes 3 aortas classified as *minimal*, 2 as *moderate*, and 1 as *severe*.

Fatty acid synthesis in liver requires DPNH and TPNH and probably occurs mostly in the extramitochondrial portion of the cell(10). Cholesterol synthesis also is dependent on a supply of TPNH. Synthesis of reduced pyridine nucleotides by "soluble" cytoplasmic enzymes of aorta, *e.g.*, LDH, G-6-PDH and 6-PGDH may be a rate limiting factor in reductive synthesis of fatty acids and cholesterol(9,10). In addition, Lehninger suggested that decreased LDH activity may be important in processes leading to calcification of the aorta(9).

Enzymatic activities based on tissue wet weight in Tables I, II and III can be directly compared with data reported by Kirk(7,8). LDH, G-6-PDH and 6-PGDH activities are remarkably similar in human and rat aortas.

Summary. (1) Extracts of arteriosclerotic aortas of female Sprague-Dawley breeder rats were assayed for lactic dehydrogenase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconic dehydrogenase. (2) Extracts obtained from moderately and severely arteriosclerotic aortas contained significantly less lactic dehydrogenase than extracts of aortas grossly normal or only slightly affected. TPNH production by combined action of glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase was significantly depressed in extracts from moderately and severely arteriosclerotic aortas. No significant differences were observed in levels of 6-phosphogluconic dehydrogenase.

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Received January 4, 1960. P.S.E.B.M., 1960, v104.

Comparison of Ultracentrifuge and Polyanion Precipitation Methods for Serum β-Lipoproteins.*† (25927)

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Isolation of human serum β-lipoproteins by precipitation of a complex with polyanions has been described by Burstein(1) and confirmed by Oncley(2), Bernfeld(3) and other authors. The polyanion precipitation method yields an insoluble complex which, in the human β-lipoprotein, can be dissociated readily (4). It has the advantage of rapid separation of β-lipoproteins from other serum constituents. This precludes exchange of lipid components between α and β lipoproteins(5) and makes possible kinetic studies of β-lipoprotein, which previously could not be done precisely due to slowness of electrophoretic and ultracentrifugal isolation technics. Completeness of precipitation technic has been checked against electrophoretic methods of β-lipoprotein isolation(6), but this comparison seems insufficient in view of poor quantitation of lipids possible by means of paper electrophoresis(7) and the ease with which mobility of β-lipoproteins can be altered by adsorbed fatty acid anions(8). We therefore studied completeness of precipitation of serum β-lipoproteins by comparison with ultracentrifugal lipoprotein analyses(9). We extended this study to include a number of animal sera, since no adequate evaluation of applicability of the precipitation method to sera of common laboratory animals has been published. Much of published work used dextran sulfate as the polyanion, but this compound is not available in this country. Mepesulfate, the sodium salt of sulfated polygalacturonic acid methyl ester methyl glycoside (Hoffmann - LaRoche,) whose use was described parenthetically by Burstein(4), is the only suitable polysulfonated macromolecule available at reasonable cost. We therefore used it and compared its action with that of a sample of rice starch sulfate prepared by Drs. T. Ushiyama and J. Mehl by a slight modification of Grönwall's method(10).

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* Supported in part by grant from U.S.P.H.S.

† Technical assistance of S. M. Velcoff is gratefully acknowledged.

Methods. Sera were obtained from volunteers or laboratory animals or purchased from Colorado Serum Co. They were cooled to 1°C before use. Rice starch sulfate was used at concentration of 1 mg/cc of serum by adding a saturated solution (10 mg/cc) in saline to the serum with constant agitation. Mepesulfate was used at level of 5 mg/cc of serum, and calcium acetate was added to make final concentration 0.17 M. Suspensions thus formed stood half hour before centrifuging. Supernatant was decanted and precipitate washed with 0.10 M calcium acetate. The precipitate was suspended in saline of density 1.063, β-lipoproteins isolated by standard ultracentrifugation technic(9) using Spinco Model E preparative ultracentrifuge, and assayed by Spinco Model L analytical ultracentrifuge. The supernatant solutions were similarly processed after adjusting them to density 1.063 with sodium chloride.

Results. For analysis of β-lipoproteins of human serum, very satisfactory results were obtained using either mepesulfate or rice starch sulfate as the polyanionic precipitating agent.

Using mepesulfate, 3 human sera were investigated, and in 2 of these no β-lipoprotein material could be detected in the supernatant remaining after precipitation, while in a third only 8% of previous β-lipoprotein level remained. The precipitate from either method was readily soluble in 1.6 M sodium chloride (density: 1.063) and lipoproteins could be recovered ultracentrifugally unchanged.

A simple screening test for β-lipoprotein levels in patients was devised, based upon precipitation of β-lipoproteins with mepesulfate and determination of protein content of precipitate with biuret reagent, after washing and defatting with dimethoxymethane. Comparison of total cholesterol level and this test yielded a correlation coefficient of 0.76 for 43 sera, indicating that the test might be of some value clinically. Correlation with other assays for β-lipoprotein is currently in progress.

TABLE I. Recovery of Lipoproteins from Human Serum.

S_f class	0-12	12-20	20-100	100-400
Whole serum	280 mg %	48	77	34
RSS precipitate	260	29	55	30
Supernate	6	2	12	10

With animal sera rather variable results were obtained. Thus, Table II lists completeness of precipitation as judged from lipoprotein analysis of sera and the supernate from precipitation and also the fraction of total β-lipoproteins recovered from the precipitate by flotation at density 1.063.

There appeared no correlation between flotation fractions and β-lipoprotein recovery after precipitation. The material lost during precipitation and dissociation procedure derived equally from all fractions.

Discussion. Precipitation of β-lipoproteins by either rice starch sulfate or mepesulfate is satisfactory with human sera and correlates very closely with ultracentrifugal isolation of the same fractions. β-lipoproteins are readily recoverable from the precipitate by dissociation of the complex in concentrated NaCl solution.

We had better results using mepesulfate as precipitating agent. It is easily used at higher concentration than rice starch sulfate because of greater solubility. With several animal sera it gave more complete precipitation of β-lipoproteins. It does, however, require addition of calcium and therefore cannot be used

TABLE II. Precipitation of Animal β-Lipoproteins.

	Rice starch sulfate		Mepesulfate	
	% precipitated	Recoverable	% precipitated	Recoverable
Calf	74	50	62	62
Cat	90	40	100	10
Chicken	42	40	90	
Ferret	99	30		85
Guinea pig	90	66	100	100
Horse	80	80	"	"
Man	91	85	98	
Monkey	86	86	100	100
Mouse	100	83	"	"
Opossum	66	4	96	
Rabbit	100	39	100	92
Rat	26	24	"	100

with plasma. In the absence of added calcium no precipitate formed and no changes in ultracentrifugal properties of β-lipoproteins occurred. Rice starch sulfate precipitates human β-lipoproteins in absence of added calcium, but for maximal precipitation of several animal β-lipoproteins calcium was required.

Quantitative evaluation of analytical ultracentrifuge patterns is fraught with difficulties which combine to cause a sizable error in determinations, particularly when lipoprotein concentrations are either very low or very high. Thus, the difference from quantitative precipitation values for human sera seen in Table I are well within the experimental error of the method.

Summary. Ultracentrifuge analysis confirms that polyanion-complex precipitation method for human serum β-lipoproteins is adequate and does not denature the lipoproteins when either rice starch sulfate or mepesulfate are used as precipitating agent. The method is applicable to isolation of β-lipoproteins from several but not all species of animals.

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Received April 29, 1960. P.S.E.B.M., 1960, v104.

Determination of Ceruloplasmin Oxidase in Serum. (25928)

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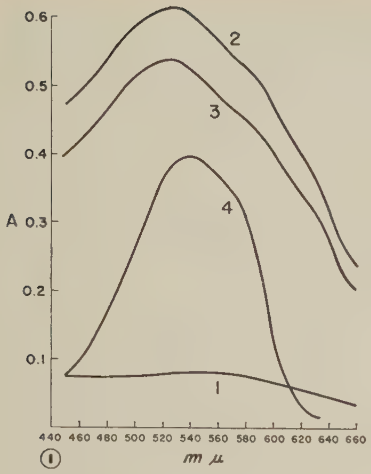
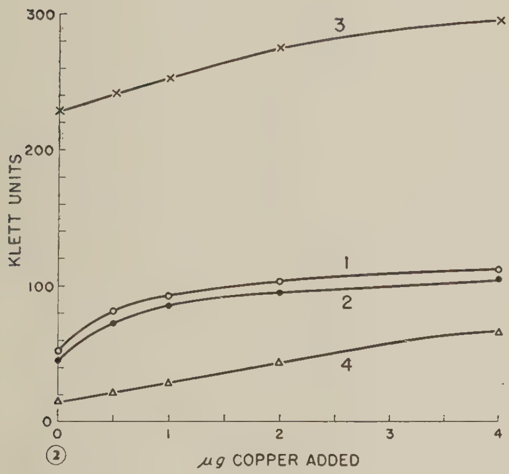
Ceruloplasmin is a copper oxidase which can be determined photometrically by rate of oxidation of p-phenylenediamine(1,2,3,4). Oxidation of p-phenylenediamine (PPD) by serum is catalyzed by serum ceruloplasmin and by copper and iron present in serum as well as in water and reagents used in the test. To determine oxidation due to ceruloplasmin alone, it is necessary either to correct for or inhibit catalytic oxidation due to metals. Both approaches have been used. In the method of Ravin(3) a serum blank is determined in which ceruloplasmin is inhibited by azide. In methods of Humoller *et al.*(2) and Broman (4), catalytic oxidation by metals is inhibited by complexing them with ethylenediaminetetraacetate (EDTA). This paper presents (a) a modification of Ravin's method which points out an error in original method, (b) a study of various factors which must be controlled in the procedure, (c) normal values obtained with this procedure and (d) results of study of validity of methods employing EDTA to inhibit metal-catalyzed oxidation of PPD.

Methods. Reagents. 1. Acetate buffer, 0.1 M, pH 6.0. Add 10 ml 0.1 M acetic acid (0.57 ml glacial acid plus water to 100 ml) to 200 ml 0.1 M sodium acetate (1.36 g $CH_3COONa \cdot 3H_2O$/100 ml). pH should be 5.95-6.00. 2. Sodium azide, 0.1% in acetate buffer. pH must be 5.95-6.00. Store in refrigerator. 3. p - Phenylenediamine · 2HCl, 0.25% in acetate buffer. Recrystallize commercial salt as follows: Dissolve in water, add Darco charcoal, warm in water bath at 60°C with occasional mixing and filter. Add acetone to filtrate until turbidity appears, refrigerate several hours, filter off PPD · 2HCl and dry the crystals in the dark in vacuum





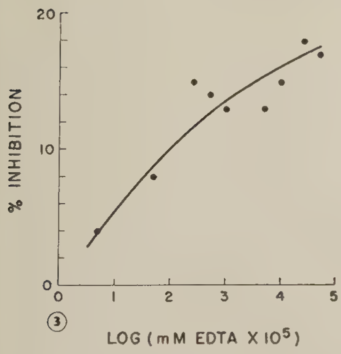


FIG. 1. Absorption curves in test for ceruloplasmin. Curve 1—blank *vs* water at 30 min. Curve 2—normal serum *vs* water at 30 min. Curve 3—normal serum *vs* blank. Curve 4—Pontacyl violet 6R, 0.0159 mg/ml of 0.5% acetic acid, read *vs* water. Perkin-Elmer recording spectrophotometer, model 4000-A.

FIG. 2. Effect of azide on substrate oxidation by added copper. Curve 1—reagent blank. Curve 2—reagent blank + azide. Curve 3—serum. Curve 4—serum + azide.

FIG. 3. Inhibition of oxidase activity by EDTA.

desiccator over anhydrous $CaCl_2$. Store in brown bottle. To prepare 0.25% reagent, dissolve 12.5 mg in 3.0 ml acetate buffer and, using narrow range pH paper, adjust pH to 6.0 by adding 1 N NaOH dropwise from 0.2 ml serologic pipet (approximately 0.1 ml required). Add acetate buffer to final volume of 5 ml. This reagent can be used to about 2 hours after preparation if kept in the dark. *Procedure with Beckman model DU spectrophotometer, equipped with thermospacers.* Compartment temperature kept at 37°C. To "blank" cuvet (1 cm light path) add 1 ml azide reagent, 1 ml buffer and 1 ml of PPD. To "test" cuvet add 2 ml buffer and 1 ml of PPD. Place cuvets in compartment and allow 5 minutes for temperature equilibration. Add 0.1 ml serum to each tube from a TC pipet. Read absorbance of "test" against "blank" at 530 mμ at exactly 10 min. and again at 40 min. after addition of serum. The unit of ceruloplasmin activity is arbitrarily defined as increase of 0.001 in absorbance in 30 min. under our conditions. Calculation: ceruloplasmin units = A_{530} at 40 min – A_{530} at 10 min.) x 1000. *Procedure with Klett filter photometer.* The "test" and "blank" are set up in double quantities in matched test tubes. Tubes are incubated in water bath at 37°C and covered by heavy black cloth to exclude light except when tubes are removed at 10 and 40 minutes for reading with Klett 54 filter. An artificial standard of Pontacyl violet 6R (Du Pont) is employed. A solution containing 15.9 mg of dye/liter of 0.5% acetic acid is equivalent to 400 units (units same as absorbance units defined above). Calculation:

$$\begin{array}{c}\text{Ceruloplasmin}\\\text{units}\end{array} = \frac{\begin{array}{c}\text{Klett reading at 40 min.}\\\text{minus}\\\text{Klett reading at 10 min.}\end{array}}{\begin{array}{c}\text{Klett reading of dye}\\\text{standard against water}\end{array}} \times 400.$$

Investigation of method. Artificial standard. Because of the complicated nature of oxidation of PPD, a standard cannot be set up with this substrate. A dye could not be found which had an absorption curve identical to that of PPD oxidized by ceruloplasmin (Fig. 1). The closest was that of Pontacyl violet 6R (Fig. 1). The artificial standard is for use with Klett 54 filter with Klett photometer and was not checked with any other filter photometer. *Inhibition of ceruloplasmin by azide.* Experiments in which serum tests were run with varying concentrations of sodium azide and read against reagent blanks without serum, revealed that complete inhibition of enzyme activity occurred in the range of 0.008 to 0.016%. Final concentration of sodium azide employed for the blank is 0.032%.

Destruction of ceruloplasmin at low pH. Erratic and spuriously low results were frequently obtained by original technic of Ravin (3) in which serum is mixed with unbuffered, unneutralized PPD reagent prior to addition of acetate buffer. Variable time of contact with this acid reagent (final pH 2.8) resulted in variable destruction of oxidase activity. In one typical experiment, periods of contact of serum with acid PPD reagent of 1 and 10 minutes prior to addition of buffer, gave ceruloplasmin results 55 and 89% lower, respectively, than result obtained by mixing serum with buffer prior to addition of PPD. This apparently constitutes a technical error in Ravin's method which is avoided in the method presented by buffering PPD.

Results. Ascorbic acid concentration and the lag phase. Evidence indicates that PPD oxidized by ceruloplasmin is again reduced by ascorbic acid present in serum until all ascorbic acid is used up(5). This produces a lag phase in the test which can reach significant proportions. Addition of ascorbic acid to serum to a concentration of 20 mg/100 ml produced a lag of about 5 minutes, during which no increase in absorbance occurred. This level of ascorbic acid is considerably higher than physiological levels. Study of approximately 100 sera by the DU method in which absorbance readings were made at 1-minute intervals for the first 10 minutes, followed by readings at 10-minute intervals up to 60 minutes, revealed that up to 10 minutes from zero time was required for rate to increase to a stable maximum. For this reason the rate in our method is determined after 10 minutes from zero time.

Effect of light. Catalytic oxidation of PPD was increased by exposure to visible light. The test, therefore, must be carried out in the dark.

Variation in pH. Rates of enzyme action were determined in 2 sera by the DU method over pH range of 5 to 7.1 (acetate buffer). Rates were calculated from 10 to 30 minute interval because of a curious phenomenon. At pH 6.0 and higher, the rates, after the lag phase, were constant from 10 to 60 minutes. At pH 5.8 and lower, however, a decrease in rate invariably occurred during 30 to 40 minute period. In most instances this did not appear to be curvilinear, but rather a sudden change to a lower but constant rate. Oxidase activity had a fairly sharp maximum at pH 6.0 with about 15% decrease at pH 5.5 and 25% at 6.5. The buffer has sufficient capacity to keep the increase in pH upon addition of even aged serum to within 0.05 pH unit of buffer pH. Optimal pH has been reported previously to be 5.5(6), between 6 and 7(1), between 5.5 and 6.0(2) and 6.0(7).

Effect of temperature. Activities of 2 sera were studied at 22, 32, 37 and 45°C by the DU method. Arrhenius plots of log of activity against reciprocals of absolute temperature gave straight lines with slopes of –3700. This gives an activation energy μ of 17,000 calories/mole and a Q_{10} between 30 and 40°C of 2.45.

Precision. Reproducibility of the method (95% limits) calculated from 4 sets of 6 replicates on normal sera was ± 4.5% (Klett method).

Normal values. Determinations (DU method) were made on 20 normal adult males and 20 normal adult females. There was no significant sex difference (t test, $P>0.05$) so values were pooled. The 95% normal limits determined by the "normal equivalent deviate method"(8), without log transformation of data, were 280 to 570 units (mean 425 units).

Stability of serum. Stability of 6 normal sera was studied at 25°C, at 4°C and in the frozen state. At 25°, 4 sera deteriorated up to

15% within 24 hours. The other 2 showed no significant decrease in 2 days. No change was observed at 4° within 2 days or in the frozen state within 2 weeks. Stability at 4° and in the frozen state has been reported previously(3,9,10).

The effect of ethylenediaminetetraacetate (EDTA). Experiments were performed by "Klett photometer method." Fig. 2 shows the effect of copper added as cupric sulfate to reagent blanks without azide (curve 1) and with azide (curve 2) and to serum tests without azide (curve 3) and with azide (curve 4). Points are averages of 4 experiments, ordinates are increase in Klett readings made against water from 10 to 30 minutes after start of reaction, and abscissae are μg of copper added to 6.2 ml of test solution. Azide had a measurable, but practically insignificant, effect on copper-catalyzed oxidation of substrate. In serum tests azide inhibited enzymatic oxidation but had no significant effect on copper catalysis. The shape of reagent blank curves is different from that of serum test curves and, without added copper, oxidation rate in reagent blanks is much higher than in tests with serum plus azide. These effects are probably due to binding of free copper by serum proteins other than ceruloplasmin(11,12). Addition of EDTA to final concentration of 0.2 mM decreased Klett units in all reagents blanks of Fig. 2 to less than 6 and in all tests containing serum and azide to less than 18. Iron added as ferric chloride to reagent blanks showed one-twentieth or less the catalytic activity of cupric ions. Azide had no effect on ferric ion activity and, furthermore, as previously reported(4), EDTA was without effect. "Blanks" without added copper or iron showed significant oxidation of substrate (Fig. 2). Analysis after wet ashing(13) indicated that copper content of a reagent blank including azide was less than 0.017 μg/ml. Normally, serum contains about 100 μg of copper/100 ml up to 10 μg of which is loosely-bound, non-ceruloplasmin copper(14,15,16). Use of such a serum in the test for ceruloplasmin adds only 0.0033 μg of copper/ml of test solution.

The effect of EDTA (disodium salt) on test for ceruloplasmin is shown in Fig. 3. Points are averages of 4 experiments. EDTA was added to both "blanks" and "tests" and "tests" were read against "blanks." The % inhibition was calculated from increases in Klett readings from 10 to 40 minutes after start of reaction. Inhibition appeared to approach a maximum of about 20%. The effect of EDTA was preponderantly on rate of color development in the "test," not the "blank," indicating an actual inhibition of enzymatic activity. To obtain an apparent inhibition, there would have to be either lower rates in the "test," higher rates in the "blank" or both. Addition of EDTA produced lower "blanks."

Discussion. Humoller *et al.*(2) used EDTA in final concentration of 0.17 mM (log (mM x 10^5) = 4.23) and read the test against a water blank. They obtained lower results with EDTA than without it but assumed that this was due solely to chelation of free metals present. Broman(4) employed EDTA in the same manner. He stated that EDTA was used in a final concentration of 4 x 10^{-5} M and presented data indicating no inhibition of purified ceruloplasmin at this concentration. Recalculation from Broman's description of his method, however, indicates that final concentration actually was 4.47 x 10^{-4} M, at which concentration his data indicated inhibition.

Our experiments led to the following conclusions: (a) reading color produced by serum oxidase activity on PPD against a serum blank containing azide is a valid measure of enzymatic oxidase activity of serum; (b) EDTA successfully inhibits catalytic oxidation of substrate by copper, presumably the predominant non-enzymatic catalyst present, but also produces significant inhibition of enzymatic oxidase activity of serum. It is not known whether EDTA forms a complex with ceruloplasmin through its copper, whether it successfully removes copper from ceruloplasmin, thus leading to inactivation, or whether inhibition is a nonspecific polyvalent ion effect(7). It has been shown that copper can be dissociated from ceruloplasmin under certain conditions(5,18,19). It is also not clear why only about 20% inhibition of serum oxidase activity could be obtained with EDTA. Presence of 2 ceruloplasmins has been demonstrated in serum(4) and it may be that one is much more resistant to EDTA than the other.

Summary. A method for determination of ceruloplasmin in serum is presented in which rate of oxidation of p-phenylenediamine is measured spectrophotometrically or photometrically. Correction for catalytic oxidation by free metals present is made by reading the test against a serum blank in which enzymatic activity is inhibited by azide. The effects of pH and temperature on the reaction have been investigated and normal values obtained by our method. Ethylenediaminetetraacetate significantly inhibits enzymatic oxidase activity of serum.

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Received March 15, 1960. P.S.E.B.M., 1960, v104.

Uptake of Glycine-1-C^{14} by Connective Tissue. III. Effects of Adrenal Hormones and Aging.* (25929)

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Changes in connective tissue constituents in response to administration of adrenal cortical hormones have been described many times. Histological studies have afforded evidence for their involvement in metabolism of skin connective tissue and granulation tissue of a number of species of animals(1,2,3,4). Metabolic and chemical studies, including those employing tracers, support the conclusion that glucocorticoids inhibit formation of connective tissue components, especially those of the ground substance(5,6,7). However, the extent to which these hormones influence metabolism of the collagenous components of connective tissue is uncertain. Perrone(8) was unable to demonstrate an effect of cortisone upon incorporation of labeled glycine into the collagen of rat skin. Robertson and Sanborn(9), however, found that cortisone reduced (and adrenalectomy increased) collagen concentration in carrageenin granulomas in guinea pigs. Like the carrageenin granu-

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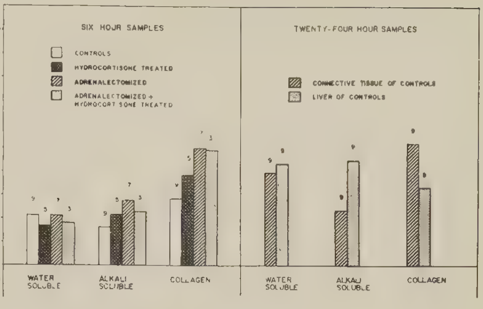
*Supported by research grants from Nat. Inst. Health, U.S.P.H.S., Bethesda, Md. For paper II in this series, see Frieden, E. H., *Endocrinol.,* 1956, v59, 69.

loma method, the sponge-biopsy technic introduced by Boucek(10) offers a means of studying connective tissue metabolism in a relatively uncomplicated system. The effects of adrenal cortical hormones upon incorporation of labeled glycine into sponge biopsy proteins are here described.

Methods. Female Sprague-Dawley rats were used. Unless otherwise stated, the animals were 4 months old and weighed about 200 g at beginning of experiment. The technic of sponge implantation was essentially that described by Boucek: Washed Ivalon sponges, measuring 2 x 2 x 1 cm, were dried at 100° for 4 hours, weighed, and moistened with sterile saline solution immediately before use. Two sponges were implanted under the skin of the back of each animal. In some animals adrenals were removed by the lumbar approach immediately before implanting the sponge. All animals received a stock diet and water *ad libitum*. Adrenalectomized animals received, in addition, 1.5% sodium chloride solution. Treated animals received hydrocortisone (0.1 ml daily of a suspension containing 10 mg/ml in sesame oil) beginning on 10th day after implantation of the sponge. On the fourteenth day,† 4 μC of labeled glycine‡ in 0.4 ml distilled water was injected intraperitoneally. The first sponge was removed after 6 hours; 18 hours later, the animals were killed and the second sponge, along with small samples of kidney, liver, spleen, and skin, was removed and weighed; after mincing or homogenizing, duplicate samples were fractionated into water-soluble and alkali-soluble fractions as previously described(11). Proteins in each fraction were precipitated 3 times by addition of TCA to 5%, washed twice with acetone, once each with ethanol:chloroform:ether (2:1:1) and benzene, and finally plated from benzene on tared aluminum planchets. The alkali-insoluble residue from each tissue sample was suspended in 5 ml water and autoclaved overnight at 15 lb pressure. The resulting gelatin solution was separated from any insoluble material and evaporated to a volume of 1 ml. Part of the solution was plated for counting; that the residue consisted entirely of gelatin, was confirmed by nitrogen and hydroxyproline determinations on aliquots of the solution. All samples were counted for a period long enough to assure precision of 5% or better; data are recorded as CPM/mg protein, corrected for self-absorption to a sample density of 5 mg/cm^2.



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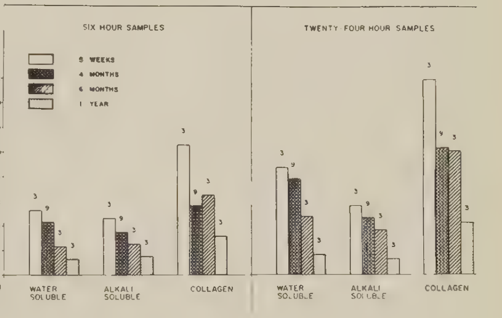

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FIG. 1. (Left) Specific activities of protein fractions obtained from sponges removed 6 hr after inj. of labeled glycine (4 μC) into control and treated animals. (Right) Specific activities of protein fractions from sponge connective tissue and liver samples removed 24 hr after inj. of labeled glycine into untreated animals. Each bar represents avg for No. of animals indicated above it.

FIG. 2. Specific activities of protein fractions obtained from sponges removed 6 hr (left) and 24 hr (right) after inj. of labeled glycine (4 μC/200 g body wt) into rats of various ages. Each bar is avg for No. of animals indicated above it.

Results. The extent of incorporation of labeled glycine during 6 hours into proteins of sponge-biopsy connective tissue is shown in the left half of Fig. 1 for 4 groups of animals (untreated controls, hydrocortisone-treated non-adrenalectomized animals, untreated adrenalectomized animals, and adrenalectomized animals which had received hydrocortisone). In the right half of Fig. 1, data after 24 hours for sponge proteins of untreated animals are compared with those for liver after the same period of time. It is apparent that uptake of glycine by connective tissue proteins of the sponge, including collagen, is quite rapid; specific activities, after 24 hours, approximate those for liver protein.

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† Preliminary studies indicated that adequate infiltration of sponge by connective tissue occurred after 2 weeks.

‡ Glycine-1-C^{14}, specific activity 3.4 mC/mM obtained from New England Nuclear Corp.

Neither adrenalectomy, nor administration of hydrocortisone, nor a combination of the 2 procedures was found to affect significantly incorporation of glycine into water-soluble sponge proteins during 6 hours (Fig. 1, left). Uptake of glycine by alkali-soluble sponge protein during 6 hours was significantly ($p = 0.01$) enhanced in adrenalectomized animals, but in neither of the other groups. Although specific activities of collagen fractions obtained from adrenalectomized (or adrenalectomized + hydrocortisone-treated) animals were considerably higher than from controls, the variations encountered make the differences of doubtful significance ($p = 0.04 - 0.2$).

Data for sponges removed after 24 hours showed no effect of adrenalectomy or hydrocortisone treatment, whether comparisons were drawn between specific activities of the various samples ($p = 0.1$ or larger) or between ratios of activity of sponge protein to that of the corresponding liver fraction.

Neuberger and Perrone(12) found that labeled glycine was incorporated much more rapidly into tendon collagen of young rats than into that of older animals. It seemed of interest, therefore, to study the effect of age of the animal upon incorporation into sponge proteins. Accordingly, incorporation of glycine-1-C^{14} into sponge proteins of rats aged 5 weeks, 6 months, and 1 year was determined. Experimental details were as in previous experiments, except that amount of glycine injected was adjusted to 4.0 μC/200 g body weight. Data for all 4 groups are included in Fig. 2.

Discussion. Our data offer no evidence for a significant inhibitory effect of hydrocortisone upon incorporation of labeled glycine into collagen of sponge biopsy connective tissue and thus are consistent with Perrone's data for skin(8). The discrepancy between our data and those of Robertson and Sanborn (9) may be more apparent than real, since an effect sufficient to reduce collagen content of the granuloma by 20% over a period of 14 days might easily escape detection in our experiments. It may be concluded that effects of adrenal glucocorticoids upon connective tissues are mediated principally through effects upon ground substance. Thus, the observation by Sobel *et al.*(7) that cortisone administration significantly reduced hexoseamine: collagen ratios in skin and other tissues of rats may be explained by a reduction in hexoseamine-containing mucopolysaccharides, without a concomitant change in collagen content.

The most interesting finding in these experiments relates to the effect of host age upon glycine incorporation into sponge proteins. There appears to be a steady decrease in rate at which glycine is incorporated from 1 month to 1 year of age (Fig. 2). This effect is not confined to collagen, but involves the water and alkali-soluble fractions as well. This fact, as well as the fact that all sponges had been in place for the same length of time before administering labeled glycine, indicates that the results cannot be due to dilution by metabolically inert collagen present in larger amounts in sponges taken from older animals.

Several possible explanations might be advanced to explain the observation of lowered glycine uptake by sponge proteins in older animals. Of these, the most likely would appear to be (a) lowered metabolic activity by connective tissue-forming cells of the host, or (b) diminished availability of substrate as a result, for example, of decreased fluid exchange between sponge and peripheral circulation. A decision between these alternatives might be made on the basis of *in vitro* studies of rate of amino acid incorporation into sponge-biopsy slices from animals of various ages.

Summary. When glycine-1-C^{14} is injected into rats bearing Ivalon sponges implanted 2 weeks previously, significant radioactivity is found in the various protein fractions (including collagen) of the sponge connective tissue

after 6 hours. Adrenalectomy at the time of implantation exerted some influence upon the extent of incorporation into some of the fractions, but administration of hydrocortisone for 2 days to either intact or adrenalectomized animals had no effect. When studies of glycine uptake by sponge-biopsy connective tissue proteins were made in rats of various ages, a steady decrease in rate of incorporation into all fractions was observed as age of the host increased from one month to one year.

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Received April 4, 1960. P.S.E.B.M., 1960, v104.

Failure of Hypophysectomy, Adrenalectomy or Thyroidectomy to Affect Response of Non-Esterified Fatty Acids to Fasting. (25930)

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Recent investigations focused attention upon the role of non-esterified fatty acids (NEFA) in energy metabolism. As a consequence of rapid turnover of this fat fraction, a flexible source of energy is made available to the organism(1). The plasma level of NEFA appears to be the resultant of rates of release from adipose tissue and rates of subsequent transport and utilization. The mechanisms which control NEFA release are imperfectly understood, though hormonal control and dietary factors have been implicated. Insulin inhibits(2) while epinephrine(3), growth hormone (G.H.)(4), and adrenocorticotropic hormone (ACTH)(5) accelerate release of NEFA from adipose tissue. All these substances are active *in vitro* when incubated with epididymal fat pads. Except for ACTH, they have also been demonstrated active in the intact animal. Dietary factors also influence plasma NEFA levels. Prolonged fasting results in increased levels which are decreased again following carbohydrate meal(6,2). In addition, increased *in vitro* release noted in adipose tissue obtained from fasting rats is decreased by adrenalectomy or hypophysectomy(7,4). These observations suggested that response of NEFA to diet is dependent upon hormonal control. The present study was undertaken to delineate the role of certain hormonal factors on *in vivo* response of plasma NEFA to fasting.

Materials and methods. Sprague-Dawley rats weighing 150-200 g were used. Hypophysectomized rats were obtained from Hormone Assay Lab. Purina chow diet and bread supplements were provided. Studies were performed approximately 2 weeks following surgery. Adrenalectomy and thyroidectomy were performed under ether anesthesia. Sham operation, simulating adrenalectomy was also performed. Animals were studied 5-8 days after operation. Adrenalectomized rats received 1% saline in place of drinking water. All animals, unless otherwise stated, were fed standard Purina chow and provided water *ad*

TABLE I. Response of Plasma NEFA to Fasting.

Animal preparation	No.	Fed *ad lib*	No.	16° fasted
Normal	41	380 ± 18.0*	11	957.5 ± 54.8
Controls	13	320		
Sham-operated	5	335		
Hypophysectomized	11	186.5 ± 16.2	8	744.8 ± 21.9
Adrenalectomized	10	226.8 ± 16.1	10	698.3 ± 38.3
Thyroidectomized	12	190.3 ± 27.1	12	674.8 ± 31.0

* NEFA in μeq/l ± S.E.M.

lib. Animals were ultimately sacrificed by decapitation. Blood was collected in heparinized tubes and centrifuged immediately at 4°C. NEFA was determined by adaptation of Dole's method(2) using 0.2 ml aliquots of plasma. Determinations were done in duplicate or triplicate.

Results. NEFA response to fasting. Forty-one normal rats, provided with food *ad lib.*, were sacrificed at various times during the day, to determine whether diurnal variation in NEFA occurs in rats. No significant difference in NEFA levels was found in rats killed between 8 a. m. and 3 p. m. An average figure of 380.5 μeq/l ± 18.0* was taken as normal plasma value for rats fed *ad lib.* The effect of varying periods of fasting was tested in 29 rats. Eleven animals fasted overnight for 16 hours, showed a mean value of 957.5 μeq/l ± 54.8. Three groups of 6 rats, fasted for 24, 48, and 72 hours respectively, showed the following NEFA levels: 846.0, 749.8 and 1004.3 μeq/l. Because of the highly significant difference in NEFA levels at 16 hours of fasting between fed and fasted groups ($p<.001$) this period of fast was chosen for study in various endocrine preparations which follow.

The effect of sham surgery hypophysectomy, adrenalectomy and thyroidectomy was studied. The results may be seen in Table I.

Examination of data by method of analysis of variance revealed that overall effect of fasting in all groups tested is highly significant ($p<.001$). In fasting groups, normal plasma NEFA is significantly higher than that in 3 treatment groups ($p<.001$) but the differences among the treatment groups are not significant. In feeding experiments, normal levels are significantly higher than those in treatment groups, though no differences exist among the treatments. No differences could be found in response to fasting in treatment groups as compared with normal.

Discussion. Our data demonstrate that hypophysectomy, adrenalectomy, or thyroidectomy results in significantly lower than normal plasma NEFA levels whether animals are fed or fasted. Despite the lower level in the fed state, the rise in plasma NEFA after fasting is of the same absolute order of magnitude as in normal animals. There was an absolute rise of 577 μeq/l in normal compared with 558, 471, and 485 μeq/l in 3 treatment groups. The per cent rise in treatment groups was significantly greater than in normal. Sham operation had no effect on NEFA levels.

These observations suggest that lower NEFA levels in endocrine operated animals are the result of deficiencies of hormonal factors. The rise in NEFA with fasting appears independent of pituitary, adrenal, or thyroid control.

It is reasonable that deficiency of G.H. and other pituitary trophic hormones such as ACTH and TSH which accelerate NEFA release might result in a lower level of plasma NEFA in hypophysectomized rats. In the thyroidectomized group, absence of thyroid hormone could explain a low NEFA level since thyroidectomy results in decreased pituitary G.H.(8), an impaired NEFA response to epinephrine(9), and thyrotoxic individuals show elevated NEFA levels(10). The effect of adrenalectomy is more difficult to explain in view of the probability that there is an increase in ACTH which accompanies this operation which might increase NEFA levels. Absence of adrenal steroids does not appear to account for low NEFA levels (unpublished observation) although absence of the adrenal medulla may be significant.

It is possible that normal NEFA response to fasting is due to the fact that decreased insulin secretion occurs with fasting. This may then be reflected in decreased utilization of glucose by adipose tissue with subsequent increase in NEFA release. This series of changes may be independent of extra-pancreatic hormonal control.

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* Standard error of mean.

Summary. There is no evidence of diurnal variation of plasma NEFA levels in rats. Hypophysectomized, adrenalectomized and thyroidectomized animals show significantly lower plasma NEFA values than normal rats either when food is provided *ad lib.* or after fasting. After 16 hour fast, both normal and operated rats show marked increase in plasma NEFA levels. It is probable that the mechanism involved in NEFA response to fasting is not controlled by pituitary, thyroid or adrenal hormones.

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Received April 11, 1960. P.S.E.B.M., 1960, v104.

Method for Determination of Phenylalanine Mustard and Related Alkylating Agents in Blood.* (25931)

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The growing interest in regional chemotherapy by perfusion wherein high concentrations of cytotoxic agents are employed makes it desirable to study reaction sites and persistence of these compounds in blood. A colorimetric procedure for p-di-(2-chloroethyl)-phenylalanine[†] (PAM) and related alkylating agents in blood has been adapted from procedure described by Epstein(1) for estimation of alkylating agents in water and non-aqueous solvents with nitrobenzylpyridine (NBP). Optimal conditions for determination of PAM were established and the revised procedure was also used for determination of HN2 and 5-(bis(2-chloroethyl)amino) uracil[‡] (U-8344). The results of these *in vitro* studies on colorimetric estimation of these alkylating agents in the presence of blood are described.

Methods. Heparinized human blood with added PAM was incubated at 37°C in a water bath. Aliquots were withdrawn at intervals, centrifuged, and the plasma collected. It was noted that blood samples containing PAM could be kept at ice bath temperature for at least 3 hours without loss of color reactivity. Solutions of PAM containing less than 1 mg/ml were made in physiological saline. More concentrated solutions were made with propylene glycol and saline or with a 25% solution of ethanol in saline. In each case a slurry was made of the PAM with a few drops of the first named solvent before the major portion of solvent was added. The mixture was warmed to 40°C in water bath to complete solution, then diluted to final volume. HN2 (Mustargen, Merck Sharp & Dohme) was dissolved in water and diluted with saline. U-8344 was dissolved and diluted in 25% ethanol in saline. The reagents used for color determination were: γ-(4-nitrobenzyl) pyridine, 5% in methyl ethyl ketone,

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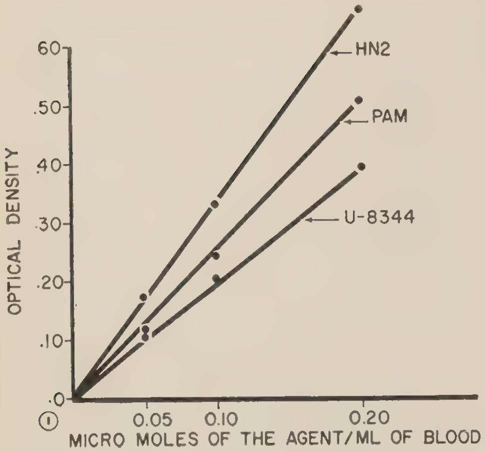
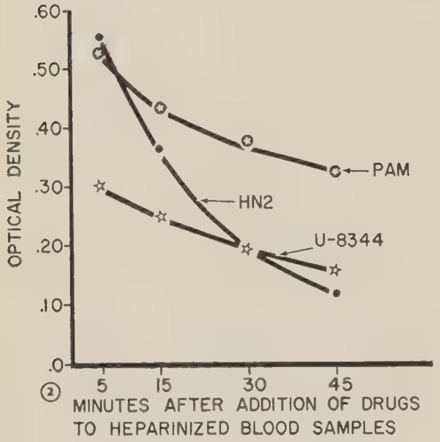
* This work supported by grant from Nat. Cancer Inst.

[†] This compound obtained through generosity of Dr. George Hitchings, Burroughs Wellcome and Co.

[‡] Kindly supplied by Dr. Hal Petering, Upjohn Co.





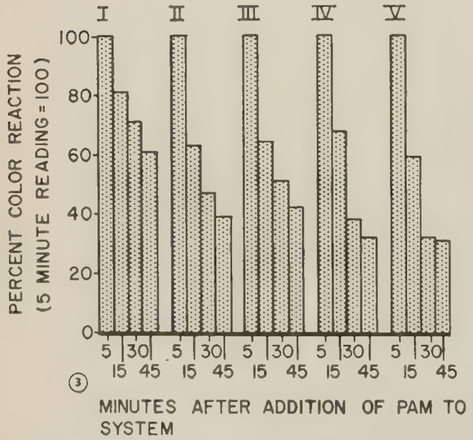


FIG. 1. Standard curves obtained by addition of NBP to alkylating drugs, PAM, HN2 or U-8344.

FIG. 2. Reaction of PAM, HN2 and U-8344 with blood as measured by color reaction with NBP. Concentration: 0.2 μmole/ml.

FIG. 3. Loss of reactive PAM from perfusion system as measured by color reaction with NBP. I. Control. PAM in heparinized blood incubated *in vitro* at 37°C. II and III. PAM in perfusion systems in patients wherein leakage was minimal. IV and V. PAM in perfusion systems in patients wherein leakage was from 25% to 30%.

triethylamine, 50% in acetone, and acetic acid, 5%. Stock solutions of PAM were made up in saline by diluting accurately weighed quantities in volumetric flasks. Aliquots were further diluted to make stock solutions to contain 0 to 0.2 μmoles/ml. One ml samples of plasma were pipetted into 12 ml centrifuge tubes followed by addition of 0.1 ml acetic acid, 2.9 ml distilled water and 0.5 ml of NBP reagent. Each tube was shaken vigorously, covered with small short-stemmed funnel, and heated 15 minutes in 80°C water bath. Tubes were then cooled in ice bath and centrifuged. Two ml of supernatant layer were transferred to a cuvette. Two ml of triethylamine color reagent were added and the sample read immediately in Bausch and Lomb Spectronic 20 colorimeter at 565 mμ employing a water blank. Absorbance of a reagent blank was determined as above and subtracted from that of sample. Amount of PAM was determined by comparing with a curve (Fig. 1) prepared by using standard PAM solutions in plasma in above procedure. The purple color formed by the reaction between PAM and NBP had a maximum absorbance of 562 mμ. For maximum color the reaction mixture should be between pH 4.5 and pH 5.0 before heating. The pH was adjusted with acetic acid. For comparison of samples, reaction mixtures should have identical pH values. The 80°C temperature was adopted in preference to boiling water because it caused less bumping of material in the reaction tube and prevented loss of sample. After heating and centrifugation, samples can be kept at room temperature for at least 1 hour without loss of color. However, measurement of absorbance immediately after addition of color reagent is imperative, as the color fades on standing. If conditions of procedure are maintained good reproducibility of absorbances is obtained.

Results. PAM was added to blood and color determinations were made at intervals (Fig. 2). A decrease of color reaction indicated that PAM reacted with a component or components of blood and was then not available for color reaction with NBP. That the colored material was formed with biologically active PAM was indicated by experiments with mice and dogs (unpublished) in which PAM was dissolved in animal's blood and reinjected at intervals. White blood cell count depression decreased with decrease of colored material formed.

The reaction of PAM, HN2, and U-8344 with blood in concentrations and at conditions comparable to those in the perfusion system as measured by color reaction is given in Fig. 2. These results show that PAM and HN2, 2 agents currently used in perfusions, react with blood at different rates and persist in blood for different periods of time, while U-8344 reacts at a rate similar to that of PAM.

Loss of color reactive PAM from blood of a perfusion system may be attributed to PAM which reacted with blood of the system and with tissues of perfused part plus amount lost *via* leakage into the general circulation (Fig. 3). The loss due to reaction with blood was rapid for about 15 minutes, then proceeded at a steady rate. In a perfusion system when PAM reacted with both blood and tissues the initial loss was greater, but the general pattern was maintained when leakage was less than 5%. When leakage was of the order of 25% the pattern changed. The color reaction 5 minutes after addition of PAM to the system (5 minutes are required for thorough mixing) corresponds to less than total amount injected. Therefore, when percentage of color reactive PAM lost from the perfusion system was calculated from residual percentage (Fig. 3,V) it was actually greater than the 70% indicated by the graph.

Summary. A procedure for estimation of PAM and related alkylating agents in blood by its reaction with NBP is given. This procedure is applicable for study of the reaction with and persistence in blood of these agents *in vitro* and in perfusion systems.

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Received May 6, 1960. P.S.E.B.M., 1960, v104.

Epithelial Changes in Vaginal Isografts in Male Mice.* (25932)

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Vaginal isografts in female mice respond normally to both intrinsic and extrinsic estrogens, although response is less regular than that of the intact organ(1). Similar isografts in male mice show cornification in response to extrinsic estrogens and mucification in response to intrinsic androgens(2,3). In castrated male mice bearing ovarian transplants, cornification of vaginal grafts occurred but cyclic activity was not demonstrated. In females, similarly prepared, however, cyclic changes have been described(3). Ovarian isografts in castrated female mice or rats show cyclic formation of vesicular and luteinized follicles but do not do so in intact or castrated males(4,5). The following report concerns our observations on periods of estrus in vaginal epithelium transplanted to castrated male mice bearing ovarian isografts.

Material and methods. Intact vaginas, removed from 16- to 20-day-old strain A female mice, were transplanted into 6 groups of hosts, each consisting of 8 male strain A mice 2 to 4 months old (Table I). Three of these groups (I,II,III) were castrated at the same time and 2 of them (I,II) received bilateral intraocular isografts of one-eighth of an adult ovary from mice 2 to 4 months old, 5 to 6 weeks later. Identical ovarian transplants were made into one (IV) of the 3 non-castrated groups (IV, V, VI). All vaginal grafts were fistulated so that smears of exfoliated

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* This work was supported by a Research Grant from Nat. Cancer Inst., U. S. Dept. of H. E. & W.

TABLE I. Mean Diameters of Vaginal Isografts before and after Estrogen Administration (If Any) to Castrated and Intact Male Mice with or without Ovarian Transplants.

Exp. group	Vaginal diameter before estrogen, mm	Wk of single estrogen dose admin.	Vaginal diameter after estrogen, mm
I Cast.; ovary	9		
II *Idem*	9	12	9
III Cast.	5	14, 15, 16	7
IV Intact; ovary	3	10, 11	6
V Intact	6		
VI "	6	8, 9, 10, 11, 12	9

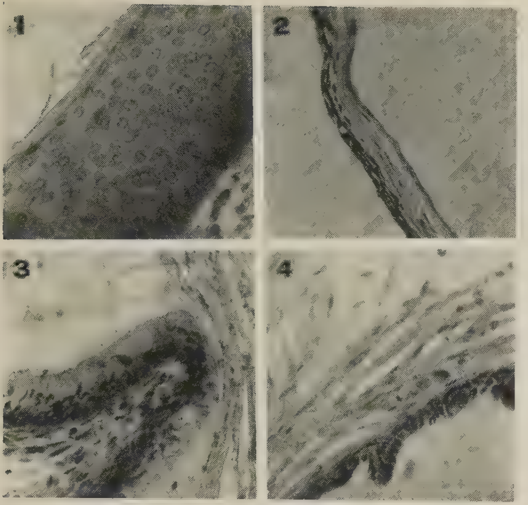
cells could be obtained (by lavage); such smears were examined for 8 to 14 consecutive days upon 3 to 5 occasions, between 11th and 30th weeks after vaginal transplantation. Twenty months after original experiment, experimental groups I, III, IV and V were duplicated and vaginal smears taken at same intervals for 17 to 56 consecutive days. At beginning of each series of examinations contents of the fistulated grafts were washed with buffered sterile saline. Representative vaginal grafts were removed for histological preparation in 19th or 20th week. All animals, except those in Groups I and V, were given subcutaneously from 1 to 5 weekly standard doses of 0.025 mg of estradiol dipropionate in sesame oil. This was done not only to determine effects of extrinsic estrogen upon size of vaginal grafts but to enlarge size of graft to facilitate preparation of satisfactory smears. The last dose of estrogen was administered during the week before grafts were prepared for taking vaginal smears, except in Group III where the last dose was given during week that preparations for smears were made.

Results. Ovarian transplants. In castrated males (I, II) the intraocular ovarian transplants, by the end of first month, occupied approximately half of the anterior chambers. Thereafter, rate of growth was retarded and, by the end of week 12, transplants occupied 70 to 90% of anterior chambers. They exhibited vesicular, cystic and hemorrhagic follicles, but, as hitherto reported(5), did not develop corpora lutea. In intact males (IV), intraocular transplants attained their maximal growth by end of first month, when they occupied 20 to 30% of anterior chambers. They, too, showed vesicular follicles regularly, and hemorrhagic follicles occasionally.

Vaginal transplant growth. By end of 12th week, vaginal transplants had attained mean diameters shown in Table I. Estrogen administration did not affect transplant size in castrated males bearing ovarian transplants but did cause moderate enlargement in castrated and intact males and marked enlargement in intact males bearing ovarian transplants. All vaginal transplants diminished in size during daily smearing procedures. Subsequently, with healing of fistulas prepared to allow smear sampling, vaginal grafts in castrated males bearing ovarian transplants (I, II) continued to dilate, reaching a diameter of over 4 cm by end of one year.

Vaginal histology and exfoliation. Castrated males with ovarian transplants (I) had proestrous smears during weeks 11 and 12. In some animals one or 2 phases of estrus (with diestrus between them) occurred following weeks 15 and 16; in others a proestrous smear was found at the same period but similar estrous phases occurred following weeks 19 and 20. From 8 to 14 days elapsed between beginning of 2 successive estrous states; full vaginal estrus itself occupied 2 to 6 days. In either case, smears had returned to proestrous (or occasionally the diestrous) phase a month later and remained so until week 30 when daily smearing was discontinued. In animals (II) that received estrogen at week 12, smears were of the estrous type at weeks 13 and 14 but reverted to proestrus and showed 1 or 2 estrous phases following weeks 15 and 16. Estrogen, in a single standard dose, was given to duplicate group I of castrated males with ovarian transplants after their reversion to proestrus or diestrus following single or repeated estrous phases. A single long period of estrus (22 to 28 days from one proestrus to the next; 1 to 17 days of full vaginal estrus) followed this administration with reversion to continuous proestrous state.

Histologically, vaginal transplants in these groups showed a typically stratified squamous epithelium with 10 to 12 layers of cells (Fig. 1) and a graft content of keratinized layers



Vaginal isografts in various types of male hosts, × 218.

FIG. 1. Castrated male bearing ovarian transplants.
FIG. 2. Castrated male without ovarian transplants.
FIG. 3. Intact male bearing ovarian transplants.
FIG. 4. " " without " " .

with some epithelial cells and leucocytes.

Castrated males without ovarian transplants (III) had a diestrous smear (although a number of epithelial cells were occasionally present) from week 19 to 24. Estrogen was administered at time of initial smearing (weeks 15 to 16) when smears disclosed a late proestrous phase. Estrogen was administered to the duplicate group, after continuous diestrus was established, at week 19, as a single standard dose. Vaginal transplants responded with a single long period of estrus (16 to 28 days from diestrus to diestrus; 8 to 20 days of full vaginal estrus), then reverted to continuous diestrus.

Histologically, the vaginal epithelium consisted of one or 2 layers of cuboidal cells (Fig. 2) with graft content of leucocytes and epithelial cells.

Intact males with ovarian transplants (IV) had a diestrous smear (except for a few animals in metestrus from the previous estrogen) at all times. Histologically the vaginal epithelium showed 2 to 4 layers of cuboidal cells (Fig. 3), the outermost being strongly mucified with occasional infiltrated leucocytes, and a graft content of epithelial cells and necrotic leucocytes.

Intact males without ovarian transplants (V) had diestrous smears, often with numbers of epithelial cells, at all examinations. Similar hosts (VI) which had received 5 preliminary doses of estrogen showed an estrous or late proestrous smear at initial examination. The duplicate group received a single dose of estrogen after establishment of a continuous diestrous smear and responded to it with a single long period of estrus, followed by reversion to diestrus.

Histologically, vaginal epithelium consisted of 2 to 4 layers of somewhat flattened cuboidal cells with occasional areas of mucification (Fig. 4). The graft contents were scanty epithelial cells with leucocytes.

Discussion. Changes in vaginal smears have not been described by other authors in castrated males with ovarian grafts(3) or in hypophysectomized males with grafted male or female pituitaries(6). However, examinations were made at one or 2 months after grafting; estrous phases in our animals were absent until 4th or 5th month after grafting. Repeated cyclic activity occurs in comparably treated females within one month. Presumably, periodicity of pituitary output of FSH, with its stimulation of estrogen secretion by transplanted ovarian tissue to a level sufficient to induce vaginal cornification, is not established in the male castrate for a relatively long period, then is only ephemeral.

The virtual absence of growth of vaginal grafts in intact males with ovarian transplants without estrogen administration, together with their histological structure, in comparison to that in intact or castrated males without ovaries, illustrates the antagonism between estrogenic and androgenic action on vaginal mucosa previously described(2,3,7,8). The relatively great amount of vaginal growth, and type of vaginal histology, in castrated males with ovarian transplants was due, presumably, to the higher output of estrogen by ovarian grafts in these hosts under the influence of post-castrational elevation of pituitary FSH output.

Summary. 1. Isografts of intact immature vaginas grew maximally in castrated male mice with ovarian transplants and minimally in intact males with similar grafts. Growth was intermediate in castrated or intact males without ovarian grafts. 2. Vaginal grafts showed one or 2 estrous phases in castrated males with ovarian grafts between 4 and 5 months after grafting but otherwise remained in late proestrus. Such activity did not occur in castrated males without ovarian transplants or in intact males with or without ovarian transplants, vaginal grafts in these hosts remaining in diestrus.

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Received May 9, 1960. P.S.E.B.M., 1960, v104.

Time Response of Lipid Changes in Cockerels after Single Dose of Estradiol Cyclopentylpropionate. (25933)

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Estrogen-induced hyperlipemia and atherosclerosis in cockerels has been described by several investigators(1,2,3,4). More recently Caldwell and Suydam(5) have studied atherogenic effect of estradiol cyclopentylpropionate (ECP) in young birds and noted significant changes in concentration of serum lipids and polysaccharides. Purpose was to study the effect of a single dose of ECP on the lipid pattern in 3-week-old cockerels. Cholesterol and phospholipids of serum, liver and aorta were determined daily, during a 7-day period, following a dose of 5 mg of ECP, to evaluate time response of cockerels to this estrogen.

Methods. Cockerels of White-Rock Broiler strain used for this experiment were obtained from a commercial hatchery when 8 days old. They were kept in thermostatic brooders and received a routine chicken starter with tap water as desired. Hormone was injected when birds were 3 weeks old. Estradiol cyclopentylpropionate (Upjohn Co.) was given intramuscularly as a single dose of 5 mg, dissolved in 0.2 ml of corn oil/bird. Beginning the next day after injection and every day for 7 days, a group of cockerels was anaesthetized, killed by decapitation and the blood collected. Untreated birds, killed on various days of this 7-day period, were considered as controls. Livers and aortas were dissected and weighed. Serum, livers and aortas were studied for lipid content. Total cholesterol was determined following the procedure of Abell *et al.*(6). For study of lipid phosphate the samples were extracted with alcohol-ether and analysis carried out following the method of Zilversmit and Davis(7). Mean values for treated and control groups were compared by the Wilcoxon Rank Test and significance levels were expressed in per cent(8).

Results. Body weight of cockerels was 226 g (200-252) at day of injection, and 387 g (343-452) at end of experiment. Injection was well tolerated and no inhibition of appetite or growth resulted. The results of study of lipids in serum, liver and aorta are presented in Tables I and II. Early and significant rise of both cholesterol and phospholipids was noted in serum and in liver after ECP. Changes in aorta were slower to appear, but significant increase of both studied lipids occurred between the 4th and 7th days, after injection of hormone.

Discussion. Significant increase of lipids

TABLE I. Response of Cockerels to 5 mg of ECP. Serum, Liver and Aorta Cholesterol. Mean ± S.E. No. of birds in parentheses.

	Total cholesterol		
Days after ECP	Serum	Liver (mg %)	Aorta
Controls			
	165 ± 4 (30)	206 ± 4 (12)	194 ± 3 (10)
ECP treated			
1	245 ± 7 (16)	*292 ± 5 (16)	192 ± 8 (10)
2	*363 ± 6 (16)	*310 ± 4 (16)	194 ± 5 (10)
3	*450 ± 12 (16)	*335 ± 8 (16)	250 ± 15 (9)
4	*542 ± 19 (16)	*371 ± 11 (16)	241 ± 17 (9)
5	*550 ± 25 (16)	*392 ± 11 (16)	*278 ± 11 (10)
6	*529 ± 22 (16)	*348 ± 9 (16)	*336 ± 13 (10)
7	*476 ± 24 (16)	*356 ± 10 (16)	*376 ± 14 (10)

* Significance level, treated *vs* control, less than 1%.

in serum, liver and aorta of young cockerels was found after a single dose of an estrogen. Rate of change was different for various tissues studied. Rise of serum and liver lipids was noted as early as 24 hours after administration of ECP. Alterations in arterial phospholipids and cholesterol developed slowly. Time element in induction of changes in the hepatic lipogenesis and cholesterogenesis was studied previously and it was found, for example, that significant changes in lipid metabolism occurred as early as 30 minutes after injection of a hormone(9). The relative rapidity in alteration of lipogenic capacity of the liver is associated with high sensitivity of this organ to changes in the level of circulating fats. The possibility exists that fatty acid synthesis in the liver is under homeostatic regulation(10). Rate of increase of serum phospholipids was not parallel with changes in liver. It is known that plasma phospholipids, being only the choline containing type, are quite different from hepatic phospholipids(11). These 2 groups of lipids may, therefore, play different roles in fat metabolism and may react in a different way to estrogenic stimulation. Changes in aortic phospholipids did not follow the rapid rise of serum phospholipids. It is apparent that phospholipids in aorta may be synthesized *in situ* and are not necessarily deposited from plasma (12). Rate of serum and arterial phospholipid turnover may be, therefore, influenced in different manner by hormones.

Several aspects of mobilization and biosynthesis of cholesterol and phospholipids remain unexplained. It is, however, evident that some hormones may influence these processes. Estradiol cyclopentylpropionate is a potent and rapidly acting stimulant of lipid metabolism under described experimental conditions. Exact function of estrogens in metabolism of lipids is not well known. This hormone may modify the activity of enzyme systems involved in absorption and incorporation of exogenous fats into tissue lipids. It may also play an important role in postabsorption phase of lipid synthesis in the tissue and in mobilization of endogenous fats. Possible effect of estrogens on cellular permeability and on transport of lipids may also be considered.

Summary. Effect of a single dose of 5 mg of estradiol cyclopentylpropionate on lipid metabolism in 3-week-old cockerels was

TABLE II. Response of Cockerels to 5 mg of ECP. Phospholipids of serum, liver and aorta. Mean ± S.E. No. of birds in parentheses.

	Phospholipids		
Days after ECP	Serum	Liver (mg %)	Aorta
Control			
	286 ± 30 (30)	2076 ± 18 (19)	704 ± 14 (10)
ECP treated			
1	*749 ± 24 (16)	*2757 ± 91 (14)	803 ± 45 (8)
2	*1467 ± 54 (10)	*2820 ± 70 (14)	740 ± 42 (8)
3	*1825 ± 73 (16)	*2850 ± 58 (16)	766 ± 97 (8)
4	*1941 ± 47 (16)	*3064 ± 32 (16)	*1013 ± 146 (8)
5	*2131 ± 99 (16)	*3091 ± 70 (16)	*1060 ± 97 (8)
6	*2378 ± 135 (16)	*2922 ± 63 (16)	890 ± 75 (8)
7	*1497 ± 107 (16)	*2899 ± 31 (16)	869 ± 73 (10)

* Significance level, treated *vs* control, less than 1%.

studied. Cholesterol and phospholipids of serum, liver and aorta were determined daily during 7 days following injection of hormone, to evaluate time response of birds to this estrogen. Early and significant rise of serum and liver cholesterol and phospholipids was observed. Increase of aortic lipids was slower to appear, but significant changes in these parameters were also noted.

Estradiol was donated by Upjohn Co. of Canada, Toronto, Ontario. A. Kangaloo is thanked for technical assistance.

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Received May 13, 1960. P.S.E.B.M., 1960, v104.

Non-Enzymatic Lysis of Fibrin *s* by Bile.* (25934)

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The fibrin plate was devised by Astrup and Mullertz(1) to serve as a simple substrate for detecting fibrinolytic activity. Lassen(2) demonstrated that heating the fibrin to 86°C for 30 minutes destroyed adsorbed plasminogen. Lysis of unheated but not heated fibrin is interpreted to indicate the presence of plasminogen activators whereas lysis of both types of plate indicates that a proteolytic enzyme is present. Using fibrin plates, Albrechtsen *et al.*(3) found plasminogen activator in many body fluids. We have been unable to find a prior study of fibrinolytic activity in bile, although Albrechtsen(4) and Todd(5) investigated plasminogen activator in body tissues and reported its absence in liver. Consequently, bile was tested on the fibrin plate and appeared to contain a heat-resistant activator. Further investigation, however, showed that the observed lysis was not due to plasminogen activation, but rather was due to simple solution of fibrin *s*(6), formed in the absence of calcium. Fibrin *i*, formed in presence of calcium, was not dissolved by bile, but was susceptible to true plasminogen activators.

Materials and methods. Human or canine bile was either tested within 2 hours of collection or frozen at –20°C for not longer than 2 weeks. Na dehydrocholate (Decholin, Endo), Na taurocholate, (Delta Chemical Works) and Na glycocholate, (Delta and Mallinckrodt Chemical Works) were used as supplied by the manufacturers. *Fibrin plates* (1,2): 0.4 g of bovine fibrinogen (Fraction I, Armour Laboratories) was dissolved in 100 ml of 0.25 M borate-saline buffer, pH 7.4(7), and filtered through Whatman #1 filter paper with suction. Eight ml was pipetted into 100 mm Kimax flat bottomed Petri dishes and clotted with 0.1 ml thrombin (Upjohn, Inc., 50 u/ml in 0.9% saline). Clots were allowed

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* Supported by research grant from Nat. Heart Inst. U.S.P.H.S.

† Work performed while a USPHS Fellow, Nat. Cancer Inst. Present address: Radioisotope Lab., Nat. Naval Med. Center, Bethesda, Md.

TABLE I. Fibrinolysis by Bile.

	Unheated plate	Heated plate
T-tube W	+++*	++
A	+	0
EW	++	0
Surgical specimens		
Cholecystitis RC	+	0
RR	+	0
Dog bile		
Meningitis	+++	0
Total body irradiation	+++	0
Human autopsy		
No gall bladder disease EB	++	0
LG	++	0
Fresh voided urine	+++	0

* + = 10-100 mm^2 of lysis
++ = 100-200
+++ = 200-300
++++ = 300-400
+++++ = >400

to stand for 10-15 minutes before testing. One half of the plates were heated at 90°C for 20 minutes. Samples were tested by delivering one drop from a 0.1 ml pipette onto unheated and heated plates. Plates were incubated at 37°C for 24 hours. Fibrinolytic activity was recorded as the product of 2 perpendicular diameters of the clear lysis zone. Standard deviation was ± 7.5%. Freshly voided urine was employed as a known plasminogen activator. *Proteolytic assays.* The caseinolytic technic of Norman(8) was utilized without modification.

Results. Bile was tested for activators on heated and unheated fibrin plates (Table I). These results suggested the presence of plasminogen activator in bile. Bile and urine were tested after heating at 60°, 80° and 100°C for one hour. The lytic agent in bile was heat stable, whereas urokinase, a known activator, was destroyed by heating.

To discover what substance in bile was responsible for lysis of unheated fibrin, 1% solutions of several bile salts, Na taurocholate, Na glycocholate, and Na dehydrocholate (Decholin), were tested and found to lyse unheated but not heated fibrin. Lysis occurred readily at 22°C and 37°C but not at 2°C, and the area of lysis depended on concentration. The lytic property of bile salts was also stable at 100°C. As these substances are “biologic detergents,” Alconox—a synthetic detergent composed of a mixture of hydrocarbon sulfonates, fatty alcohol sulfates, and complex phosphates—was tested and was a potent lytic agent similar to bile salts.

Bile salts and Alconox uniformly failed to activate proteolytic activity of plasminogen when tested by the caseinolytic technic. Bile salts did not cause delay of clotting of whole blood or produce subsequent clot lysis; Alconox, however, caused hemolysis and an infinite clotting time.

Urea dissolves fibrin *s*, formed in absence of calcium or fibrin stabilizing factor. Upon dialysis to remove urea the clot reforms(9, 10). Thirty per cent urea produced lysis on our plates, indicating presence of fibrin *s*. Heated fibrin *s* plates were not lysed by urea.

Plates prepared with 0.01 M $CaCl_2$ were subjected to 30% urea, bile, and bile salts (Table II). Addition of calcium prevented non-enzymatic lysis of fibrin but did not interfere with plasminogen activators. T-tube bile “W” was contaminated with bacteria and proteolysis was probably due to microbial growth.

Mullertz has reported that fibrin plates formed in the presence of citrate are more sensitive to enzymes than plates free of citrate(11). Because Armour fibrinogen contains 40 to 50% sodium citrate it became important to demonstrate whether the effects noted also occurred with citrate-free fibrin. Citrate was removed from a fibrinogen solution by dialysis for 24 hours against borate buffer with a change of buffer at 12 hours. Plates formed with citrate-free fibrinogen

TABLE II. Fibrinolysis with and without Calcium.

	No calcium	0.01 Ca	Heated
5 M urea	++	0	0
T-tube A	+	0	0
” W	+++	+++	+++
” EW	+	0	0
GB bile EB	+	0	0
10% desiccated ox bile in H_2O	+++	0	0
1% glycocholate	++	0	0
1% taurocholate	++	0	0
1% Alconox	+++	0	0
Urine	+++	+++	0

were sensitive to bile salts, detergent and urea and the effect was blocked by 0.01 M Ca.

Dialysis of lysed clots to remove the bile salt or detergent caused the clot to reform, demonstrating that the fibrin had been dissolved and not digested. Six ml of fibrinogen was clotted and then dissolved with 10 ml of 10% (W/V) bile salts or Alconox. The solutions were dialyzed at 2°C against 0.2 M phosphate buffer, pH 7.4, for 16 hours. At least partial recovery of the clot was evident in all cases, and both citrated and non-citrated clots could be recovered by this method.

Discussion. Olesen reported that bile salts activated fibrinolytic activity in guinea pig serum subjected to precipitation at pH 5.4 in presence of these salts(12). This appears to be a different phenomenon than we are reporting, because the bile salts required the presence of something in serum for fibrinolytic activity. The fibrin plates used by Olesen were not sensitive to direct application of bile salts although they did not have added calcium. This may represent an unexplained difference in fibrin prepared by a different method. Reconstitution of fibrin clots after dissolution by bile salts and detergent seems to be unequivocaal evidence that fibrin *s* was not digested enzymatically, but was dissolved, as it may be by a number of reagents: urea, lithium chloride, lithium bromide, sodium bromide, sodium iodide, guanidine hydrochloride, histamine dihydrochloride, and monochloroacetic acid(13). Whether the above reagents, bile salts and detergents all dissolve fibrin *s* by the same mechanism is undetermined. Only bile salts appear in physiological fluids in sufficient concentration to be confused with plasminogen activators in fibrin plate testing.

Fibrin *i*, formed in presence of calcium and fibrin stabilizing factor (FSF), is a copolymer of fibrin and FSF and is resistant to solubilizing reagents(14). It is suggested, therefore, that fibrin *i* be used in tests for enzymatic fibrinolysis to obviate artefacts such as that seen with bile. Bovine Fraction I (Armour) contains FSF so that addition of calcium during clotting gives fibrin *i*(13).

Summary. Bile salts and detergents cause "lysis" of fibrin *s* by a simple solution of fibrin. Fibrin *i* is not dissolved by bile salts and detergents. Fibrin *i* should be used in fibrinolytic assays for plasmin or plasmin activators.

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Received May 20, 1960. P.S.E.B.M., 1960, v104.

Detection and Titration of *Staphylococcus aureus* Agglutinin in Serum.* (25935)

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During studies to demonstrate alteration of metabolism of recently isolated virulent strains of *S. aureus* in presence of serum, an interesting sedimentation pattern suggestive of agglutination was observed. Quantitative differences in capacity of various sera to produce this distinctive agglutination pattern were noted, hence the phenomenon seemed worthy of study. This report presents (a) a description of this phenomenon and its quantitation, (b) evidence supporting specificity of agglutination reaction, (c) staphylococcal agglutinin titers of sera from different species, (d) agglutinin titers of pooled human gamma globulin samples and (e) preliminary data on serologic responses of rabbits and humans to vaccination with an *S. aureus* vaccine.

Materials and methods. *S. aureus* antigen is prepared by inoculating 100 ml of trypticase soy broth (TSB) with a loopful of cells from a trypticase soy agar slant that had been incubated for 16-18 hours at 37°C. The broth culture is incubated for 5 hours at 37°C, then distributed in 1.0 ml amounts into rubber-stoppered vials and stored at –20°C. The standard antigen dilution used is initially determined by pooling the contents of 2 vials and preparing serial 10-fold dilutions in TSB. One-tenth ml of each dilution is added to ten 13 x 100 mm tubes followed by 1.1 ml of TSB. Cultures are incubated overnight at 37°C and antigen titer recorded as the reciprocal of the 50% viability endpoint (VE_{50}) according to the method of Reed and Muench(1). One hundred VE_{50} is the antigen dilution used to determine agglutination response. Once standardized in this manner, titers of stock antigens remain unchanged at –20°C for periods of at least 5 months. In determining agglutination response, the appropriate antigen dilution is made in TSB from pooled contents of 2 vials and viability (VE_{50}) titer confirmed in each test. The strain of *S. aureus* selected as antigen is apparently not critical since a number of *S. aureus* isolates (serotypes I and III) have been used as antigens with good agreement as to agglutinin titers determined. Recently, an antibiotic resistant strain of *S. aureus,* readily cultivated in TSB containing 100 units of penicillin and 100 μg of streptomycin per ml, has been employed as antigen. Use of this organism facilitates the procedure by lessening risk of contamination as test tubes stand open while reagents are delivered with automatic pipettes. Each serum is heated at 56°C for 30 minutes preparatory to testing. Serial 2-fold dilutions (usually starting at 1:100) of serum are made in TSB. One-tenth ml of each serum dilution is placed in a 13 x 100 mm test tube and 0.1 ml of antigen added. Mixtures are shaken and incubated for 1 hour at room temperature after which time 1.0 ml of TSB is added to each tube. For purposes of control, the titer of a standard pool of human serum is determined each time. Variations in titer of the control serum have not been observed in excess of 2-fold on different days. Mixtures are incubated for 16-18 hours at 37°C, then read by inspection. The ease with which the end-point is distinguished is illustrated in Fig. 1. The agglutinin titer is expressed as the reciprocal of highest dilution of serum causing complete agglutination of the antigen.

Results. Antigen specificity. To validate the specificity of this reaction, a number of adsorption procedures were carried out. If the reaction is specific, adsorption of antibodies by cells of *S. aureus* should cause a quantitative decrease in titer of adsorbed serum. Accordingly, a cell suspension was prepared and aliquots were mixed with (a) human serum; (b) a prepared pool of commercial human gamma globulin; and (c) rabbit serum, from animals in which antibody for-

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* This study was made possible by Fund for Research Therapeutics.

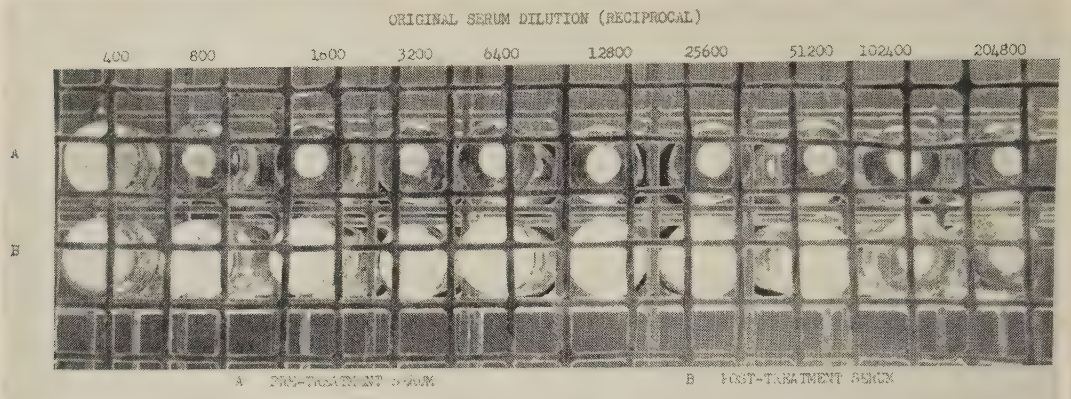

FIG. 1. Agglutination pattern of *S. aureus* in presence of rabbit serum.

mation had been induced by injection with antigen. These mixtures were placed in a refrigerator at –4°C and allowed to stand overnight. Cells were removed by Seitz filtration and sera tested for agglutination capacity by the procedure described above. Reduction of titers, 8 to 32 fold, indicates that adsorption occurred. Adsorption tests carried out with aliquots of the same sera and *S. albus* cells showing no reduction in titer offer further evidence of specificity of this reaction. Use of *S. albus* as antigen in the agglutination test was not successful. The distinctive sedimentation pattern was not observed: all tubes in the series had the same type of diffuse growth.

Agglutinin titers of animal sera. It has been shown in the past that certain animal sera show antibodies to staphylococci, hence it seemed purposeful to determine whether agglutinin titers could be demonstrated in randomly selected animal sera† by this test. Table I indicates presence of measurable agglutinin titers and also a wide range of titers within each species. It is open to speculation whether these titers reflect differences in exposure to staphylococcal infection or differences in capacity to form antibodies. Similarly there is a range of titers in human sera, and preliminary data indicate no difference in titers between persons who have been free of recognized staphylococcal infection for long periods and those who have had recent skin infections due to staphylococci. Of particular interest was the specimen of human serum which had a titer of less than 200. This serum was submitted as an unknown‡ but after decoding it was found that this specimen had been obtained from a patient with agammaglobulinemia. In light of this observation it is intriguing to speculate about the cause of the value of less than 200 in the specimen of goat serum.

Agglutinin titers in human gamma globulin: The finding of insignificant titers of agglutinin in the specimen of agammaglobulinemic serum suggested the testing of pooled human gamma globulin. Use of a living staphylococcus as the antigen made it requisite to obtain gamma globulin specimens prior to addition of preservatives.§ Variation in titer of agglutinins in the various lots is shown in Table I. These values so distinctly higher than those encountered in single samples of human sera that have been tested, suggested that the agglutinins measured are concentrated in the fraction of blood containing the presently recognized antibodies, thus indirectly supporting the view that it is an antibody that is being titered.

Serologic response of rabbits to treatment with staphylococcal vaccine. Rabbits naturally have agglutinin titers as measured by

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† The authors wish to acknowledge their thanks to Samuel V. Scheidy, D.V.M., Univ. of Pennsylvania School of Vet. Med. for these specimens.

‡ Kindly forwarded to us by George G. Jackson, M. D., Univ. of Illinois, Chicago.

§ The authors are indebted to Lederle Laboratories Division, American Cyanamid, Pearl River, N. Y. and Merck Sharp and Dohme Labs., West Point, Pa., for cooperation in making these samples available.

TABLE I. Staphylococcal Agglutinin Titers of Sera of Various Species.

Agglutinin titer (reciprocal)	Rabbit	Horse	Cat	Goat	Cow	Sheep	Man	
							Sera	γ-globulin
>409600			1	1	1			
409600								
204800			1					2
102400					1	1		1
51200		2		1	2			
25600				1		1	3	
12800		1	1				3	2
6400		2	1		1	2	17	
3200	1	1			1		11	1
1600	2					1	17	
800	4						5	
400	4			2		1	2	
200								
<200				1			1	

this test (Table I). It seemed of interest to determine whether these titers could be increased by treatment of rabbits with a staphylococcal vaccine. Accordingly 4 albino rabbits were bled, then treated intravenously with a series of injections of bacteriophage-lysed *S. aureus* vaccine. In graded doses at intervals of approximately 4 days over a period of 33 days each rabbit received 80 ml of staphylococcal preparation. Post-treatment sera were obtained from the rabbits one week after completion of treatment. All serologic responses were determined in a single test and post-treatment sera showed a 2 to 64-fold increase in titer.

Serologic response of patients to vaccination. Twenty-one male patients, all of whom had suffered from recurrent furunculosis for a period of many months, were vaccinated subcutaneously with a bacteriophage-lysed *S. aureus* vaccine.|| The arbitrary schedule of vaccination was 0.05 ml initially, 0.1 ml 4 days later and thereafter increments of 0.1 ml at 4-day intervals, up to 0.5 ml per injection. Treatment at this level was then continued weekly. Local reaction at sites of injection as minimal, the usual reaction being redness less than 3 cm in diameter, with little or no induration. Serum was obtained from each patient immediately before and 4 weeks after beginning of vaccination. Serologic responses of these patients were determined simultaneously in a single agglutination test (Fig. 2). Although there was a therapeutic response in all of these patients, only 13 of 21 patients (62%) responded with at least a 4-fold increase of titer. A therapeutic and serologic comparison of staphylococcal vaccines and toxoids will be presented later.

Discussion. It is well known that the staphylococcus is rich in antigenic substances although there is no ready means of determining the exact number(2). However, there has been a significant lack of evidence to correlate any of the demonstrated antibodies with protection or immunity against staphylococcal infection. Elek(2) pointedly emphasizes the shortcomings of previous work in correlating staphylococcal diseases of animals or man

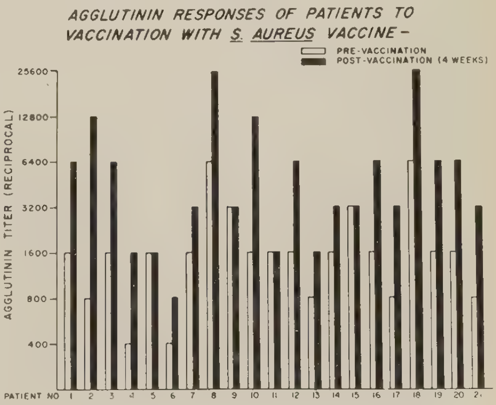

FIG. 2.

|| This preparation was supplied by Delmont Labs., Swarthmore, Pa.

with the immunologic findings and states that "Staphylococci isolated from lesions are by no means always agglutinated by the patient's serum and the demonstration of agglutinins is of no clinical importance." Without in any way wishing to challenge this statement this paper presents what we believe to be a new,** technically simple and exquisitely sensitive agglutination test, accidentally discovered in our laboratory.

The basis of the test described here is alteration of the characteristic growth pattern of coagulase-positive staphylococci in fluid medium in presence of serum from either human or animal source. It would appear that the agent responsible for this alteration of growth possesses certain generally accepted properties of antibody: a) stability in serum heated at 56°C for 30 minutes; b) semi-quantitative removal by adsorption with homologous (coagulase-positive) but not with heterologous (coagulase-negative) staphylococci; and c) an increase in titer following treatment with a staphylococcal vaccine.

The antibody measured appears to be present naturally in sera of animals and man in varying amounts. Since staphylococci are ubiquitous and there is increasing documentation of staphylococcal infections in animals it is suggested that the antibodies reflect previous experience with this organism. This statement is not necessarily in conflict with the common failure of anti-hemolysin, anti-leucocidin or agglutinin titers to distinguish patients who have staphylococcal infection. This seeming paradox may be due to the unique histopathology of staphylococcal infections.

It has, however, been possible to show an increase of titer in rabbits and in man following treatment with a staphylococcal vaccine. The vaccine employed was a bacteriophage-lysed preparation without preservative and certainly contained a) components of culture medium, b) metabolites of staphylococci, c) soluble residues of lysis of cell walls and d) living bacteriophage particles. It has been impossible to characterize the specific antigen or antibody controlling the altered agglutination pattern which is the basis for the test presented here. The serum factor does not appear to be an antibody to alpha hemolysin or bacteriophage.

The finding of insignificant amounts of the agglutinin in a single agammaglobulinemic serum and the variable, but high titers, of agglutinin in pooled, human gamma globulin support the concept that the factor we are measuring is found in that fraction of blood which contains all meaningful antibodies as we know them today. However, the antibody or agglutinin measured by this new test has not been studied extensively enough to permit an opinion as to clinical significance or its identity with a "protective" antibody. The agglutination test is so simple in its performance, so exquisite in sensitivity and reproducibility that it is presented for trial by other investigators.

Summary and conclusions. A technically simple agglutination test for detection and titration of an unidentified anti-staphylococcal factor, presumably antibody, in human and animal sera is described. By this test variable titers have been found to occur naturally in sera of animals and of man. Agglutinins are present in large amount in pooled human gamma globulin and in insignificant amounts in a single agammaglobulinemic serum. Titers of this factor can be increased by treatment of both animals and man with a staphylococcal vaccine. The suggestion is made that this factor is an antibody on the basis of a) its presence in antibody bearing fraction of blood b) its heat stability c) ability to adsorb it semiquantitatively with coagulase-positive staphylococci and d) ability to elevate titers in rabbits and man by use of staphylococcal vaccine as antigen. Preliminary observations do not enable us to identify the factor being measured by the agglutination test nor assess its clinical significance.

** We have not found any similar test in our search of the literature.

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Intracerebral Growth and Treatment of Leukemia L1210. (25936)

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Our preliminary studies of disposition of leukemia cells (L1210) in different tissues of DBA/2J and CDBA[†] mice, implanted subcutaneously with the tumor, have shown that the brain becomes infiltrated 1-3 days prior to death.[‡] It was interesting to determine whether intracerebral (I.C.) route of inoculation of lymphoid leukemia (L1210) would lend itself to studies such as: 1) Time required for disease to become systemic. 2) Degree of leukemic infiltration in different brain areas. 3) Effect of drug therapy when there is high concentration of leukemic cells in the brain, when ability of the drug to penetrate blood-brain barrier could be of considerable importance. Data are presented showing that intracerebrally injected leukemia (L1210) grows in the brain, infiltrates diffusely in the arachnoid, and rapidly becomes systemic. Significant increases in survival time were obtained with drug therapy.

Materials and methods. 10-12-week-old DBA and CDBA male mice[§] weighing 22 to 28 g were employed. For each experiment a DBA mouse bearing subcutaneous (S.C.) implant of leukemia (L1210) in the right inguinal region was sacrificed by cervical dislocation on 9th day after inoculation.[‖] A midline incision was made and the diaphragm exposed and transversely cut. Blood was collected by cardiac puncture into heparinized syringe. One volume of whole blood was diluted with 9 volumes of sterile saline (0.9% NaCl). DBA or CDBA mice were injected intracerebrally by penetrating the skull with 25 gauge needle in area of midbrain and slowly introducing 0.05 ml of inoculum, using a microliter syringe (Hamilton Co.). In comparative studies separate groups of mice were inoculated S.C. in the right inguinal region with the same volume of inoculum. Brain and spleen tissue, removed from either I.C. or S.C. injected mice, was prepared as a mash. The tissue was inserted into 1 or 2 ml syringe and forced through 20 gauge needle into equivalent volume of sterile saline. The mash was passed back and forth through the needle into syringe barrel until uniform tissue suspension was obtained. Whole blood was collected by cardiac puncture as previously described. Sterile saline was used for all dilutions.

Results. Table I shows that 3 days after inoculation of leukemic blood into the brain, tumor cells were in sufficient concentration that S.C. inoculation of 0.1 ml of 2% brain inoculum resulted in tumor growth and death in recipient mice. Leukemic titer in brain increased so that by fifth and eighth day after injection of leukemic cells into the brain, dilutions of 0.4 to 0.02% of brain suspension were capable of eliciting tumor growth and subsequent death.

Comparison was made of time required for the disease to become systemic, when I.C. and S.C. routes of inoculation of leukemia (L1210) were employed (Table II). Two groups of CDBA[¶] mice were implanted, I.C. or S.C., with 0.05 ml of a 10% concentration of leukemic whole blood, prepared as previously described. On days indicated following inoculation, I.C. and S.C. implanted mice were sacrificed; whole blood, brain, and spleen tissue were taken and used for subcutaneous inoculation into CDBA mice.

Results from Table II show that spleen was sufficiently infiltrated to transmit the disease at 2 days following I.C. injection, and that leukemic cells were present in circulating blood by the sixth day. In contrast, by the

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[*] U. S. Dept. of H. E. W.

[†] (BALB/cAn x DBA/2J)F_1 hybrid male.

[‡] Chirigos, M. A. (unpublished data).

[§] Obtained from NIH breeding colony.

[‖] Median survival time (MST) for these mice is usually 10-13 days.

[¶] CDBA male mice are susceptible to growth of leukemia (L1210), Goldin, *et. al.*(1).

TABLE I. Potency of Inoculum Prepared from Brain Tissue of Mice Inoculated Intracerebrally* with Leukemic Blood (L1210).†

	Day intracer. inoculated mice were sacrificed for brain tissue inoculum					
	Day 3		Day 5		Day 8	
% conc. of inoculum‡	Dead/Total	Range of death (days)	Dead/Total	Range of death (days)	Dead/Total	Range of death (days)
50	3/3	12–14	3/3	12–13	3/3	14
10	”	”	”	13	”	15–16
2	”	13–16	”	15–17	”	16–19
.4	0/3		2/3	16–18	”	17–20
.08	”		1/3	18	”	17–22
.02	”		0/3		1/3	20

* Median survival time of intracer. inj. mice was 9 days.

† DBA/2J mice were used throughout experiment.

‡ All inocula diluted with sterile saline and inj. subcut., 0.1 ml/mouse, in right inguinal region.

sixth day, leukemia was not transplanted successfully with blood, spleen, or brain taken from mice inoculated by the S.C. route; however, by eighth day they were capable of transmitting the disease.

Pathology. Sections of central nervous system were examined in DBA mice sacrificed on eighth day following intracerebral injection of leukemia (L1210). Diffuse infiltration by leukemic cells in the subarachnoid spaces was noted (Fig. 1). Small nodules of leukemic cells were seen in the ventricles, and occasional intracerebral blood vessels were surrounded by a cuff of leukemic cells.

Amethopterin and 3′,5′-dichloroamethopterin have been shown to prolong the lifespan of mice with advanced leukemia (L1210)(1) when the S.C. route of tumor inoculation was employed. Although leukemic cells are present in the brain late in the course of the disease, following S.C. inoculation, they are apparently not in as high concentration as when inoculated I.C.

It was of interest to determine whether treatment with these drugs could increase survival time of mice inoculated I.C. with leukemia (L1210). Results of a representative experiment are summarized in Table III. Drug therapy was begun on seventh day after mice were injected I.C. with leukemic blood, since biological evaluation of brain tissue from I.C. injected mice and degree of infiltration in brain as observed microscopically indicated that the brain is well infiltrated by this time. In this experiment, since all controls succumbed, the higher inoculum was at least 10 times greater than that required to give 100% deaths from tumor. Although there were no

TABLE II. Disposition of Leukemia in Tissues of Mice Inoculated Intracerebrally or Subcutaneously with Leukemia (L1210).*

	Day tissue inoculum was prepared from mice													
Inoculum†	Day 2		Day 4		Day 6		Day 8		Day 10		Day 11		Day 12	
	D/T‡	R‡	D/T	R	D/T	R	D/T	R	D/T	R	D/T	R	D/T	R
							Intracerebral mice§							
Blood	0/3		0/3		3/3	14–15	3/3	12	1/1	9	No intracer. survivors on days 11 and 12			
Spleen	2/3	14–16	3/3	14–16	”	10–11	”	8–9	”	7				
Brain	”	14–16	”	11–12	”	9–12	”	8–10	”	9				
							Subcutaneous mice‖							
Blood	0/3		0/3		0/3		3/3	14	3/3	10–14	3/3	11–12	2/2	9
Spleen	”		”		”		”	10–12	”	8	”	8	”	7
Brain	”		”		”		”	14–17	”	12–13	”	12	”	11

* CDBA mice were used throughout this experiment.

† 0.1 ml of undiluted whole blood, .2 ml of a 50% concentration of spleen or brain implanted subcut. in right inguinal region.

‡ D/T = Dead/Total. R = Range of death in days.

§ Median survival time, 10 days.

‖ ” ” ” , 13 ” .

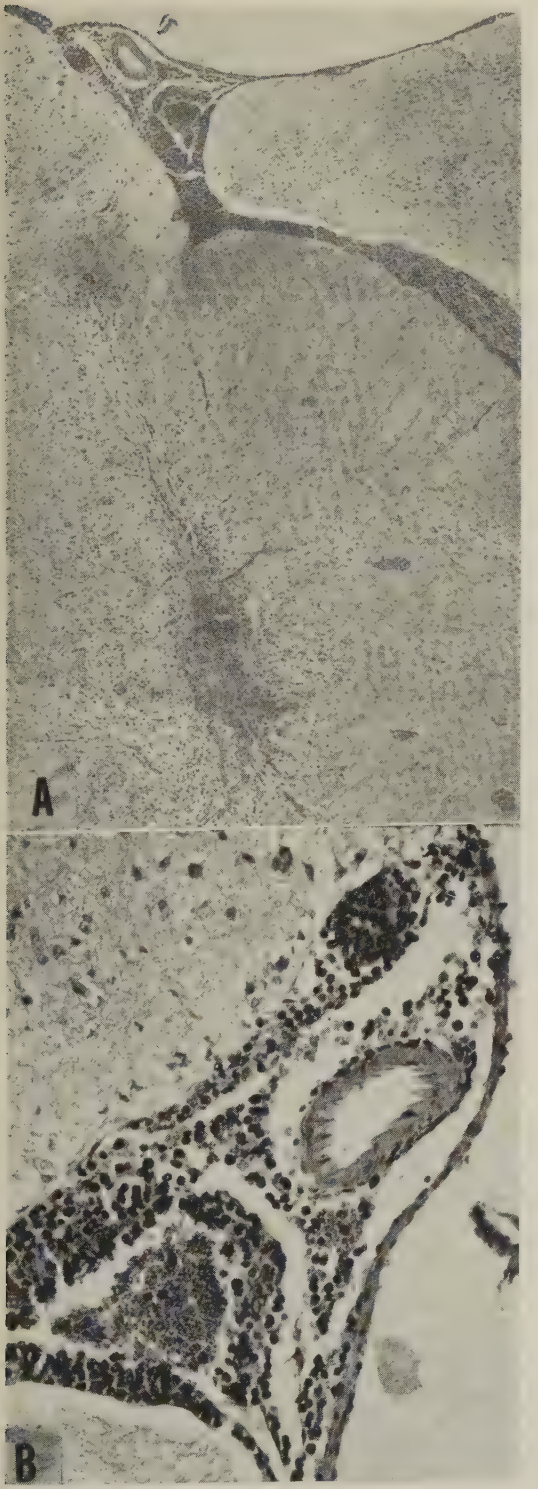

FIG. 1. (A) Photomicrograph of midbrain of mouse inj. intracer. with leukemia L1210. Needle puncture tract in midbrain is hemorrhagic and surrounded by inflammatory cells. No leukemic cells noted along needle tract. There is diffuse leukemic cell infiltration in arachnoid and a few intracerebral vessels exhibit perivascular cuffing. 40 ×.

FIG. 1. (B) Leukemic cells fill arachnoid spaces on surface of midbrain and infiltrate wall of vein. 178 ×. Stain, hematoxylin-eosin.

TABLE III. Effect of Drug Therapy on Mice Implanted Intracerebrally with Leukemic Blood (L1210).*

Conc. of blood inoculum (%)†	Drug treatment‡	Median survival time (days)	Range of death (days)
10	DCM	41	15–68
"	MTX	19	15–30
"	None	12	11–12
1	DCM	52	14–66
"	MTX	28	23–29
"	None	12	12–13

* DBA/2J mice; 10/group.

† All inocula diluted with sterile saline, and inj. intracer., .05 ml/mouse.

‡ DCM = 50 mg/kg 3′, 5′-dichloroamethopterin.
MTX = .75 mg/kg amethopterin.

Drug therapy was initiated on 7th day after mice were implanted intracer., and administered subcut., daily, in axillary region.

survivors in the treated groups, the results show that the antifolic agents caused substantial increases in survival time with both levels of inoculum.

Whether earlier treatment of I.C. injected mice would have led to complete regression of leukemia in brain has not been determined.

That both drugs were effective indicates that these drugs may be capable of penetrating the blood-brain barrier. Studies are presently being conducted with this system to determine penetrability of these and other agents known to be effective against leukemia (L1210).

Summary. Mouse leukemia (L1210) was grown successfully when introduced by intracerebral route. Brain tissue was rapidly infiltrated and the disease became systemic 2-3 days following I.C. inoculation. Deaths occurred somewhat sooner than when the disease was inoculated S.C. Administration of antifolic agents (amethopterin and 3′,5′-dichloroamethopterin) increased survival time.

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Received May 23, 1960. P.S.E.B.M., 1960, v104.

Routine Direct Measurement of Arterial Pressure in Unanesthetized Rats.
(25937)

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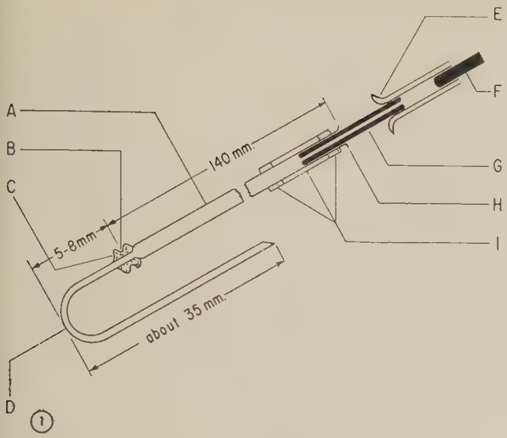
Difficulties and possible inaccuracies associated with existing indirect methods for determining blood pressure routinely in rats (lack of agreement with direct pressure measurements, requirements that animals be heated or anesthetized, errors due to size and placement of occluding cuffs) have been summarized(1,2,3). To circumvent these difficulties, we have adapted for routine use the method of permanent aortic intubation first described by Still *et al.*(2,4). Still's original method involved inserting a small polyethylene tube into the lumen of the aorta and leading it beneath the skin to an exit on the back of the neck. The free tip was then heat sealed and cut off for each measurement. We have made 3 modifications: (1) employing hypodermic needle tubing to bring the tube through the skin, (2) using a wire to plug the free end of polyethylene tubing, (3) using small size polyethylene tubing only for insertion into the aorta, the remainder being a larger size. The needle tubing allowed easy replacement of the end piece of polyethylene as it stretched and frayed with use; while larger tubing allowed systolic and diastolic pressures to be measured.

Methods and materials. Construction of the tube to be implanted involves fabrication of 3 items: (1) a "J"-shaped piece of polyethylene tubing (passing from aorta to back of the neck), (2) a piece of hypodermic needle tubing (which exits through the skin), and (3) an end piece of polyethylene stiffened by a jacket to resist mechanical trauma (Fig. 1). The tube is implanted as follows. Make a transverse skin incision about 15 mm behind the ears in anesthetized rats at least 200 g in weight. Open the abdomen on the midline, remove viscera and cover with gauze. Using a dry gauze sponge, wipe the aorta clean in the region of the bifurcation. Pass a 14 cm hubless trocar (made from 16 ga needle tubing with blunt end enlarged to about 2.5 mm o.d. by a drop of solder) through the psoas muscles lateral to left genito-femoral nerve and medial to left ureter, then on to the incision in neck. Using a few mm of PE 50, couple aortic tube and attached needle tubing to a 16 cm length of 22 ga needle tubing, insert into the trocar as far as possible, and pull through the tissues to neck incision. Thrust a needle through the skin in the midline about 6 mm in front of incision. Using the needle as a guide, pass needle tubing through the skin. Slip on the reinforced PE 50 "end piece" and attach to a tube leading to a syringe filled with isotonic saline. Using silk sewing thread (size A), make a single stitch through neck muscles, tie a square knot thus forming a loose loop anchored to the neck, then tie securely to the polyethylene tubing. Close neck incision. Anchor polyethylene tubing alongside the aorta just above the bifurcation by one stitch of the attached silk thread through psoas muscles. Cut the tip of the PE 10 on a bevel at a point slightly below renal arteries and fill tubing with saline. Occlude the aorta with the tip of a finger and puncture with a 26 ga needle about even with the anchor stitch. Withdraw needle and insert polyethylene tubing. Although seldom necessary, bleeding may be controlled by a wisp of cotton-type oxidized cellulose and gentle pressure. Replace viscera and close abdomen. No anticoagulant is necessary in the tube. About 80% of intubations were successful. Since troublesome infections occurred occasionally, we sterilized instruments and materials in a 1:1000 solution of benzalkonium chloride. Procaine penicillin G 40,000 units and dihydrostreptomycin 50 mg (as 0.2 ml, i.m., of Dihydrocillin, Upjohn) were given postoperatively. Rats must be caged individually. Blood pressure was measured using a Statham P23G pressure transducer (Statham Instruments, Hato Rey, Puerto Rico) and a Grass Model 5 polygraph



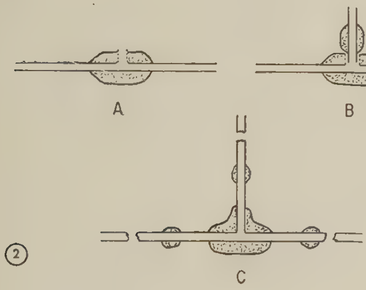


FIG. 1. Polyethylene tubing and accessories for aortic intubation. A. PE 50 tubing, 140 mm length for rats to 350 g (Clay-Adams, N. Y.). B. 4-O silk suture tied between ridges for anchoring tubing alongside aorta. C. PE 50 sealed to PE 10 [Insert pipette probe wire (Fisher 6-214, ground smaller at tip if necessary) into lumens, melt together by rotating slowly against pointed tip of moderately hot soldering iron (Ungar 4035, No. 332 tiplet, operated at about 70 volts). Melt again on PE 50 to form second ridge.] D. PE 10 fixed into "J"-shape (form loosely around glass rod and dip into boiling water). E. PE 50 with PE 160 protective jacket (hold near flame to seal together). F. 7 mm wire plug made from 18 ga needle stylet, inner end bluntly tapered (cut and grind this wire and needle tubing using dental separating disc with stream of water playing continuously on both sides). G. 20 mm long 22 ga stainless steel needle tubing with smooth rounded ends. H. PE 50 projecting beyond PE 50-160 seal flared by heating near a small flame. Tubing anchored to neck muscles by tying in the groove between flare and seal. I. PE 160 jacket 20 mm long sealed at both ends over PE 50 as in C above using a 21 ga needle stylet to maintain lumen.

FIG. 2. Preparation of polyethylene "T"-tubes. A. Insert wire (21 ga needle stylet) into lumen of PE 50, thicken wall by rotating against chisel tip (Ungar No. 333) of moderately hot soldering iron. Drill hole (No. 60) for side arm. B. Make similar thickening, cut one end very short, insert stub into hole, insert wire into side arm, and seal by rotating thickened joint against pointed tip, taking great care not to touch the thin walls of the tubing. C. Attach additional lengths of PE 50 to the arms of the "T" by melting together over a wire.

(Grass Instrument Co., Quincy, Mass.). The transducer was arranged with side outlet connected to controlled air pressure for calibration. A 3-way stopcock on the recording outlet allowed saline to be injected into the rat. Connection between rat and transducer was by a length of PE50 tubing with a piece of 22 ga needle tubing on the end. The rat was immobilized by wrapping in a towel with head exposed, the end piece of polyethylene was occluded by grasping with a shod hemostatic forceps, the wire plug removed, the tube connected to transducer and flushed with about 0.2 ml of isotonic saline, then B.P. recorded continuously for at least 1 minute. Average pressure could easily be estimated. If the tube seemed obstructed, saline was forcibly injected using a 0.25 ml syringe. At other times, it was necessary to massage the aorta gently through abdomen. Presumably, the tip of the tubing was lying against the wall of the aorta or partially occluded by a small piece of fibrin. Aortic thrombi and aneurisms have been rare since the tube was anchored alongside the aorta as described. Rats so prepared have been used for as long as 6 months. We have used this method routinely in B.P. studies for over a year and have prepared at least 300 rats. Drugs may be injected into the aorta with only a few seconds interruption of pressure recording. A "T"-joint was fabricated in the PE 50 tubing leading from transducer to rat (Fig. 2) and the side-arm of "T" connected through a small stopcock to a syringe. After closing the connection to the transducer, drugs were injected, then flushed into the animal by about 0.1 ml of saline from the syringe on the transducer.

Discussion. Although we have routinely recorded mean arterial pressures (by electronic damping of the pulse pressure), systolic and diastolic pressures may also be measured. The same values were obtained when, in an anesthetized rat, pressures were recorded simultaneously from one carotid artery directly to a transducer using a 20 ga needle as a cannula, and from the other carotid artery using a complete aortic tube assembly connected to a second transducer. In the latter however, the contour of the pulse wave was rounded.

Normal mean arterial pressure, based upon 44 Wistar origin females, age 2 to 5 mo., 3 successive measurements per rat on different days, averaged 129 mm Hg. Analysis of variance showed the rat to rat standard deviation (square root of rat component of variance) to be 7.7 mm Hg, and day to day standard deviation on a given rat to be 6.2 mm Hg(5). Most of our experience has been with Grollman type(6) renal hypertensive rats, in which B.P. as high as 300/200 have been recorded. We have considered rats as hypertensive when their mean B.P. was over 150 mm Hg, most of them ranging between 160 and 200 mm Hg.

Reliability of readings from the photoelectric tensometer(7) were studied by simultaneous recording of direct aortic B.P. Our tensometer, built within The Upjohn Co., recorded cuff pressure and light transmission through the foot simultaneously on a paper chart. Personal bias was minimized by accepting all clear-cut readings. A cuff as designed by Rosett and Handler was used(2). Five successive readings were made, the cuff removed and replaced in apparently the identical manner, and another 5 readings made; 4 such series an each of 10 different rats. Actual B.P. from the aorta varied only a few mm of Hg. Tensometer B.P. agreed well within the 5 replicate readings when the cuff was undisturbed (average range 25 mm Hg), but there were often large variations between averages of replicate series on the same rat (up to 70 mm Hg, average range of series means 40 mm Hg), which could be due only to cuff placement. That the presence of the tube in the aorta might have altered blood flow to the legs, and so caused such variations, seems doubtful since a similar series using a polyethylene tube tied into a carotid artery showed the same discrepancies. (Carotid cannulated preparations remained functional only a few days.) Within the limits imposed by cuff placement variation, tensometer and direct aortic systolic pressures agreed.

Summary. Method is described whereby polyethylene tubes, permanently implanted in aorta, may be used routinely for repeated direct measurement of arterial pressure in unanesthetized rats.

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Received May 24, 1960. P.S.E.B.M., 1960, v104.

Influence of Certain Basic Compounds on Conversion of Mevalonic Acid To Cholesterol by Liver Homogenates.* (25938)

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Evidence has been presented that a polyanion is essential for maintenance of ATP levels in whole rat liver homogenates incubating aerobically(1). The "natural" polyanion in this system appears to be RNA(2) but other polyanions such as DNA, polyethylene sulfonate, and heparin may substitute for it(3). ATP levels are not maintained in the presence of the *polycation* protamine presumably because of formation of an anion-cation complex that renders the essential polyanion unavailable(4). Additional data are presented here concerning the specificity of the polycation as an inhibitor of the system essential for

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* Supported by research grants from Nat. Science Fn. and Nat. Inst. of Health and funds through State University of N. Y.

maintenance of ATP levels in aerobic homogenates.

Methods and materials. The biosynthetic experiments involved preincubation of 5 ml aliquots of a 200 xg supernatant fraction of rat liver prepared as previously described(1-4) with 5 ml amounts of solution containing 1 mg ATP, 10 mg DPN, 100 mg of sodium succinate, and various amounts of protamine or other compound studied. Each flask was aerated with a stream of oxygen and preincubated with agitation for 30 min at 37°. Following preincubation each flask was opened, 1 ml DL MVA-2-C^{14} solution added (500 γ, 50,000 cpm), the contents aerated, and the flasks then reincubated for an additional 3 hrs. After final incubation homogenates were saponified, extracted with petroleum ether, ether extracts dried with sodium sulfate, filtered, evaporated, taken up in scintillation mixture and counted. Reasons have been presented previously for a working hypothesis that conversion of mevalonic acid to nonsaponifiable material (NSF, largely cholesterol) under the experimental conditions described is a measure of ATP level existing at the time homogenates are supplemented with mevalonic acid(1). The triethylenemelamine (TEM) used was kindly made available by Dr. E. L. R. Stokstad, Lederle Laboratories, American Cyanamid Co. The trypsinized protamine was prepared by treating protamine in aqueous solution or in 0.1 M pH 7.0 phosphate buffer with 5% of its weight of crystalline trypsin for 18 hrs at 37° followed by heating at 100° for 15 min to inactivate trypsin. The other compounds studied were readily available commercial preparations.

Results. The data obtained are summarized in Table I. Inhibitory activity of protamine (MW 10,000 or less) in the system studied is markedly reduced by digestion of the protein with trypsin. Polylysine (MW 4,000) is even more inhibitory than protamine. Bovine serum albumin (MW 67,000) with an isoelectric point (4.8) considerably lower than that of protamine(10-12) is not inhibitory. Similarly, biosynthesis of NSF from mevalonic acid was not influenced by predigestion with such small molecular weight bases as putrescine, spermidine, spermine, arginine, or TEM.

TABLE I. Biosynthesis of NSF from Mevalonic Acid by Liver Homogenates as Influenced by Preincubation with Certain Basic Compounds.

Exp.	Material tested		cpm
1		None	8114
	1 mg	protamine	8060
	2		8065
	5		7936
	10		26
	20		25
	1	polylysine	7386
	2		9337
	5		32
	10		22
	20		33
2		None	7509
	5 mg	protamine	8314
	10		48
	20		34
	5	trypsinized protamine*	6694
	10		7264
	20		84
	5	bovine serum albumin	7050
	10		7274
	20		7763
3		None	5711
	4 mg	protamine	6052
	8		21
	12		22
	20		21
	4	trypsinized protamine†	6187
	8		8403
	12		5978
	20		3484
4		None	9503
	5 mg	protamine	9980
	10		31
	20		26
	5	putrescine	8660
	10		8362
	20		7328
	5	spermidine	9037
	10		8285
	20		7028
	5	spermine	9386
	10		9652
	20		9045
	5	arginine	9708
	10		9089
	20		9937
5		None	6037
	1 mg	TEM	5642
	2		5969
	5		5902
	10		5675
	20		5282

* Protamine trypsinized in water solution.

† Protamine trypsinized in 0.1 M pH 7.0 phosphate buffer.

Discussion. The results obtained are consistent with an hypothesis that a polyanion essential for maintenance of ATP in aerobic homogenates of liver is made unavailable by high molecular weight polycations. The combination of large molecular weight and frequent positive charges seems essential for activity since (a) partial hydrolysis of protamine by trypsin lowers inhibitory activity, (b) bovine serum albumin exceeding protamine in molecular weight but containing few positive charges is not inhibitory, and (c) compounds such as putrescine, spermidine, spermine, and arginine comparable to protamine as bases but of relatively low molecular weight are not inhibitory. Apparently TEM under the conditions studied does not alkylate enough groups to reduce the effectiveness of the essential polyanion.

Summary. Conversion of mevalonic acid to non-saponifiable material by rat liver homogenates under conditions where conversion is a function of ATP level existing at time of mevalonic acid addition was inhibited markedly by preincubation with protamine or polylysine. Inhibition was less or absent following preincubation with trypsinized protamine, bovine serum albumin or a number of small molecular weight bases. The results are interpreted as indicating that a *combination* of high molecular weight and strong basicity are essential for inactivation of a polyanion previously shown to be essential for maintenance of ATP levels in liver homogenates.

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Received May 31, 1960. P.S.E.B.M., 1960, v104.

Investigation of Rose Bengal Conjugation.* (25939)

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Rose Bengal (tetraiodotetrachlorfluorescein) is one of the many dyes utilized in evaluation of liver function. Although uptake, storage and excretion of Rose Bengal I^{131} by the liver have been studied extensively, little is known of the metabolic processes involved. Clearance of Rose Bengal by the liver is impaired by concurrent administration of bromsulfalein (BSP)(1-4), and possibly by bilirubin(5). Recent studies have shown that hepatic clearance of BSP and of bilirubin involves conjugation with cysteine/glutathione (6,7) and glucuronic acid(8-10), respectively. Such observations suggest that these pigments may compete with Rose Bengal for initial uptake or subsequent metabolism by the liver and therefore that the competition may take place at a conjugation step. Attempts to demonstrate the presence of conjugates of Rose Bengal in biological fluids constitute the subject of this report.

Methods. Fresh human bile was obtained from 3 subjects with presumably normal liver function by means of T-tubes inserted in the common bile duct. After collection of control samples, 6-10 ml of Rose Bengal (Matheson Co.) (8.9 mg/ml) was administered intravenously; bile was collected at 30 min. intervals for 2 hr thereafter. For isotopic studies, 0.1 mC of Rose Bengal I^{131} (Robengatope, Squibb) was added to each dose before injection. Serum and urine samples were obtained from an additional subject with obstructive jaundice 45 min after injection of Rose Bengal. Control specimens were prepared by adding Rose Bengal *in vitro* to bile, serum and urine in concentrations approximating those

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* Supported in part by U.S.P.H.S. grant.

[†] U.S.P.H.S. medical student part-time summer fellow, 1959.

TABLE I. Mobility of Pure and Secreted Rose Bengal after Paper Chromatography.

Solvent	Ratio v/v	R_f		
		Rose Bengal bile	Control bile*	Standard*
Tert butanol/water	5.9 :1	3	3	3
	4.7 :1	14	13	14
	3.7 :1	18	18	18
	2.6 :1	32	32	32
	1.72:1	64	63	64
	1.68:1	67	67	67
	1.1 :1	81	81	80
	.78:1	100		100
Tert butanol/water (ammonia atmosphere)	1.9 :1	90	90	90
Tert butanol/water (acetic acid atmosphere)	1.9 :1	50	50	50
n-butanol/ethanol/water/NH_4OH	5:.05:1:2.2	65	64	69, 42
n-butanol/acetic acid/water	4:1:2.7	100		100
1% NH_4OH saturated with isoamyl alcohol		61	59, 38	63, 38
25% ethanol/5% NH_4OH	1:1	70	72	82
Methanol/water	1.63:1	70	74, 70	83, 74

* Second value = secondary band.

found in the experimental samples. All samples were frozen and stored at −10°C until used. Rose Bengal was extracted and extraneous proteins were precipitated from bile, urine and serum by the addition of 2 volumes of acetone. As an alternate procedure on bile samples, 0.1 N silver nitrate was used as a precipitant and the pigment was redissolved in a concentrated sodium chloride solution. Bile was initially purified in paired alumina columns (1 x 5 cm). Activated alumina (Alcoa) was suspended in 90% isopropyl alcohol containing 0.13% ammonium acetate and packed by gravity. Acetone extracts of bile dried *in vacuo* were dissolved in a minimum volume of the packing solution and added to the columns. Rose Bengal was eluted in 80% isopropyl alcohol/NH_4OH (200:1). Bile extracts containing Rose Bengal I^{131} were initially purified in similar paired alumina columns using acetone/water (20:1 and 10:1) for loading and acetone/water (8:1 and 7:1) for elution of pigment. Ascending paper chromatography was performed on all samples before and after deproteination and on bile at each step of purification, using Whatman 3 MM paper and various solvent systems as listed in Table I. Mobilities of Rose Bengal from experimental and control fractions were compared by visible color and by fluorescence with a short wave ultraviolet Woods lamp (model SL 2537, Ultraviolet Products, Inc., San Gabriel, Calif.). Chromatograms of Rose Bengal I^{131} were cut into 1 cm strips which were counted in a well-type scintillation counter. Paper electrophoresis, using 0.25 M acetic acid or 0.25 M ammonium hydroxide, was performed on acetone-eluted column fractions from bile. To study the nature of Rose Bengal concentrated in the liver, 0.6-1.6 mg of the dye per 100 g of body weight was injected into the inferior vena cava of anesthetized rats. Animals were sacrificed 10-20 min after injection, the liver removed, and the pigment extracted by homogenizing the tissue in 5 volumes of acidified methylethyl ketone. Lipids were removed on the column by ether and toluene, and pigment was eluted in the isopropyl-alcohol system. Similar extracts, prepared by homogenizing the tissue in acetone/water were subjected to paper chromatography. Mercaptide conjugation was investigated in the perfused isolated liver system described by Brauer, *et al.*(11) and by Plaa(12). After a 1 hr equilibration period, 51 μC of cystine-S^{35} was added to the perfusion media; bile samples were taken at 30 min intervals. One hr later, 23.2 mg of Rose Bengal was added; bile collection was continued. Control and experimental samples, each containing an equal number of counts per total volume, were extracted and purified in paired alumina columns. Column fractions of Rose Bengal bile, purified by successive

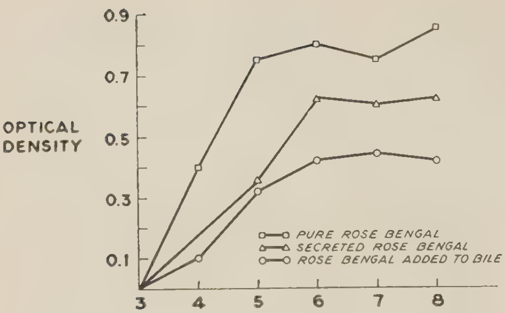

FIG. 1. Effect of pH on absorption of pure and secreted Rose Bengal at 545 mμ (unequal dye concentrations were used).

paper chromatography, were tested against internal standards for glucuronides by the Dische reaction(13).

Results. Absorption spectra at 545 mμ of pure Rose Bengal were obtained at pH 3-8 in citric acid-disodium phosphate buffers (Fig. 1). Absorption increased with increasing pH until about pH 5. The pH of maximum absorption of secreted Rose Bengal in purified bile fractions was displaced to pH 6. Identical displacement, however, was observed with control samples of bile containing added Rose Bengal.

Chromatography and electrophoresis. Secreted Rose Bengal in bile moved primarily as a single, visible fluorescent spot which could not be differentiated by extensive combinations of chromatographic systems (Table I) or by electrophoresis from Rose Bengal added to bile *in vitro*. When bile obtained after administration of Rose Bengal I^{131} was chromatographed in 6 different *tert* butanol solvents, mobilities of the visible pigment and radioactivity correlated well over a wide range of R_f values.

Rose Bengal in serum, urine and liver. Rose Bengal obtained from the serum and urine of a subject with biliary obstruction or extracted from rat liver was indistinguishable from pigment added *in vitro* to comparable control specimens.

Conjugation with cysteine or glucuronic acid. Rose Bengal and isotope appeared rapidly in bile following perfusion with cystine-S^{35} and dye. After purification by column and paper chromatography, the Rose Bengal fraction from both experimental and control bile contained only trace amounts of label. Determination of glucuronide content of highly purified extracts of bile showed that not more than 14% of the Rose Bengal could have been conjugated with glucuronic acid.

Discussion. The fact that secreted Rose Bengal was indistinguishable from pigment added to control bile and that significant levels of non-pigmented products were not observed after chromatography of secreted Rose Bengal I^{131} indicates that the pigment need not be metabolized on its passage through the liver. It is unlikely that metabolites of Rose Bengal are formed but subsequently degraded in bile since none could be detected either in the serum of a patient with an obstructed common bile duct, where regurgitation of the metabolites would be expected to occur, or in extracts from rat liver obtained shortly after injection of the dye. Under both circumstances, BSP metabolites have been reported (14). It is emphasized, however, that the negative nature of these results does not rule out the possibility that secreted Rose Bengal is modified but that the modified form could not be detected by the chromatographic systems employed in this study.

In contrast to BSP(6), cystine-S^{35} was not incorporated into secreted Rose Bengal. Therefore, competition of the 2 dyes for excretion does not occur at the step of mercaptide conjugation in the liver. Also, failure to discover a Rose Bengal glucuronide suggests that the pigment does not compete with bilirubin at the level of glucuronide conjugation.

Uptake and excretion of pigments by the liver may involve 2 independent mechanisms (15,16). Recent experiments as yet unpublished have shown that BSP is taken up and concentrated in the liver as the free pigment prior to its ultimate conjugation as a mercaptide. Conceivably, competition of various pigments may occur at this early uptake step (17) and thus be comparatively independent of the routes required for subsequent conjugation.

Summary. 1. Metabolites of Rose Bengal could not be demonstrated in human bile, serum or urine or in extracts from rat liver by a variety of systems involving paper and column chromatography and electrophoresis.

2. Conjugation with cystine-S^{35} or glucuronic acid was not demonstrable. 3. It was concluded that Rose Bengal is not conjugated by the liver in the same form reported for bilirubin (glucuronides) or bromsulfalein (mercaptides).

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Received June 10, 1960. P.S.E.B.M., 1960, v104.

Synthesis and Some Pharmacological Properties of a Peptide Derivative of Oxytocin: Glycyloxytocin.* (25940)

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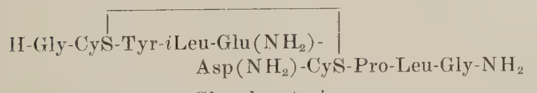
In a series of studies on the relationship between structure and biological activities of the posterior pituitary hormones and their analogs and derivatives, we became interested in the biological properties of peptide derivatives of these hormones, *i.e.* compounds in which the hormones remain unchanged except for the attachment of additional amino acids at the N- or C-terminal position. As a comparatively simple derivative of this type in which the amino group of oxytocin is involved in peptide linkage, glycyloxytocin has been synthesized.



$\text{H-Gly-}\overline{\text{CyS-Tyr-}i\text{Leu-Glu(NH}_2\text{)-Asp(NH}_2\text{)-CyS}}\text{-Pro-Leu-Gly-NH}_2$

Glycyloxytocin

Method of synthesis. The protected decapeptide, carbobenzoxyglycyl - S - benzyl - L - cysteinyl - L - tyrosyl - L - isoleucyl - L - glutaminyl - L - asparaginyl - S - benzyl - L - cysteinyl - L - prolyl - L - leucylglycinamide, which was employed as the intermediate for the glycyloxytocin, was prepared from the protected nonapeptide previously used for the synthesis of oxytocin(1), namely S-benzyl-N - carbobenzoxy - L - cysteinyl - L - tyrosyl-L - isoleucyl - L - glutaminyl - L - asparaginyl-S - benzyl - L - cysteinyl - L - prolyl - L - leucylglycinamide. For the present experiments, this protected nonapeptide was prepared by the stepwise synthesis recently described by Bodanszky and du Vigneaud(2). To obtain the protected decapeptide, the carbobenzoxy group was first removed from the protected nonapeptide by treatment with HBr in acetic acid and the resulting hydrobromide of the S,S′-dibenzyl derivative of reduced oxytocin

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* This work supported in part a grant from Nat. Heart Inst., U.S.P.H.S.

(dibenzyloxytoceine)† was converted to the free base and allowed to react with *p*-nitrophenyl carbobenzoxyglycinate. Removal of the carbobenzoxy and benzyl groups from the protected decapeptide by treatment with sodium in liquid ammonia yielded the decapeptide in its sulfhydryl form, and oxidation of the latter compound by aeration gave glycyloxytocin. The observation was made that in the preparation of glycyloxytocin, oxidation to the disulfide requires a much longer time than in the case of oxytocin. In the preparation of oxytocin, 2 to 4 hours of aeration at pH 6.5 are usually sufficient to cause the disappearance of the reaction with sodium nitroprusside for sulfhydryl groups, whereas 4 days of aeration were consistently necessary for glycyloxytocin. This marked difference in the speed of the disulfide bond-forming reaction suggests that formation of the 20-membered ring may be hindered by the presence of the glycyl residue. Glycyloxytocin was subjected to countercurrent distribution in the system butanol-ethanol-0.05% acetic acid (4:1:5) for 950 transfers at which point the main component was obtained in a peak with a partition coefficient (K) of 0.36. The experimental curve plotted from the Folin color values was in agreement with a theoretical curve calculated for this K value. The glycyloxytocin obtained after evaporation and lyophilization of the solution showed a single ninhydrin spot on paper chromatography. Analysis of the material on the starch column showed the constituent amino acids to be present in the expected ratios, and the elementary analytical values were also satisfactory.

Pharmacological tests. Glycyloxytocin was tested for avian depressor activity in comparison with a laboratory standard of highly purified oxytocin by measuring the drop in blood pressure of the chicken by the method of Coon(3), as modified by Munsick *et al.*(4). The glycyloxytocin in sufficient amount did produce a drop in blood pressure, but the variation in relative responses to oxytocin and to glycyloxytocin was so great from bird to bird as to preclude a definite assignment of units per mg of avian depressor activity for the derivative. Furthermore, the character of the response to glycyloxytocin differed from that to oxytocin. The drop in blood pressure after administration of oxytocin is usually complete within 10 seconds and a return to the normal level usually takes place within another 10 to 20 seconds. The response to glycyloxytocin is slower, about one minute being required for the pressure to reach the lowest level, and the return to normal level is much more gradual. Occasionally the blood pressure does not return to its original level. The ratio between the amount of oxytocin and glycyloxytocin necessary to produce comparable drops in blood pressure varied from 1:170 to 1:650 in different birds. After injection of glycyloxytocin the response to oxytocin or glycyloxytocin was greatly decreased. In fact, 0.68 μg of glycyloxytocin, which in itself produced no noticeable change in blood pressure, inhibited by 50% the normal response to oxytocin when injected simultaneously with 0.04 μg of oxytocin and had the same effect on subsequent doses of this amount of oxytocin administered 10 minutes later. When larger doses of the derivative were injected, almost complete inhibition of the effect of subsequent doses of oxytocin resulted. The inhibitory effect lasted for some hours, the exact length of time depending on the amount of glycyloxytocin injected. At higher dose levels the inhibitory effect lasted at least 6 hours. Obviously, further studies into the nature of the response are necessary for a full understanding of the behavior of this compound in the bird.

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† In the original report of this compound by Gordon and du Vigneaud (PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 723), it was simply referred to as S,S′-dibenzyloxytocin, since it seemed obvious that it would be interpreted as the dibenzyl derivative of the reduced form of oxytocin. However, it now seems advisable to have a specific name for the reduced form in the nomenclature of the rapidly expanding number of compounds related to oxytocin and vasopressin. We therefore suggest the name *oxytoceine* for the reduced form of oxytocin and *vasopresseine* for the reduced form of vasopressin. This nomenclature would be analogous to that of such sulfhydryl compounds as cysteine and homocysteine. The dibenzyl derivative of reduced oxytocin would then be called *S,S′-dibenzyloxytoceine* and the corresponding derivative of reduced vasopressin, *S,S′-dibenzylvasopresseine.*

In the assay for pressor activity(5) in the male rat, 120 μg of glycyloxytocin exhibited a pressor effect equivalent to 0.018 μg of arginine-vasopressin and 1.2 μg of oxytocin, respectively. The character of the response again differed from that observed with both arginine-vasopressin and oxytocin in that the rise was much more gradual, taking 10 minutes to reach its peak and from 30 minutes to 1 hour to return to its former baseline. With both vasopressin and oxytocin the rise in blood pressure in the rat and the return to normal are complete within 15 minutes. At these dosages the glycyloxytocin had no inhibitory effect on the pressor response of oxytocin or arginine-vasopressin, whether the derivative was injected simultaneously or following injection of the hormone.

When assayed for oxytocic activity by the method of Holton(6) on isolated rat uteri, 1.7 μg of glycyloxytocin showed a potency equivalent to that of 0.004 μg of oxytocin. The dose-response curve of glycyloxytocin appeared to be somewhat different from that of oxytocin, the linear portion of the curve having a shorter range than that of oxytocin. No inhibitory effect on the uterine-contracting effect of subsequent doses of oxytocin was detected.

Experimental.‡ *p-Nitrophenyl Carbobenzoxyglycinate.* Carbobenzoxyglycine (10.4 g) and *p*-nitrophenol (8.4 g) were dissolved in ethyl acetate (150 ml) and treated with dicyclohexylcarbodiimide (10.3 g) at 0°. After 1.5 hours at room temperature the N,N′-dicyclohexylurea was filtered off and the solvent was removed from the filtrate *in vacuo*. The residue was recrystallized from ethanol (100 ml) and the crystals were washed with ethanol (50 ml); wt. 13.8 g, m.p. 126-128°. A recrystallized sample melted at 128°; lit. m.p. 128°(7), 124-125°(8).

Carbobenzoxyglycyl - S - benzyl - L - cysteinyl - L - tyrosyl - L - isoleucyl - L - glutaminyl - L - asparaginyl - S - benzyl - L - cysteinyl - L - prolyl - L - leucylglycinamide. The protected nonapeptide, S-benzyl-N-carbobenzoxy - L - cysteinyl - L - tyrosyl - L - isoleucyl - L - glutaminyl - L - asparaginyl - S - benzyl - L - cysteinyl - L - prolyl - L -leucylglycinamide (2.6 g)(2), was dissolved in acetic acid (20 ml) and 3 *N* HBr in acetic acid (40 ml) was added to the solution. After 1.5 hours at room temperature the hydrobromide was precipitated with ether (400 ml), filtered off, and washed with ether. After being dried briefly over calcium chloride and sodium hydroxide at room temperature *in vacuo* the hydrobromide was dissolved in dimethylformamide (25 ml) and treated with triethylamine (3 ml) and *p*-nitrophenyl carbobenzoxyglycinate (0.80 g). After one day at room temperature the reaction mixture was diluted with ethyl acetate (300 ml) and the precipitate was washed with ethyl acetate (100 ml), ethanol (200 ml), and again with ethyl acetate (50 ml); wt. 2.71 g, m.p. 242-245° (dec. 246°), $[\alpha]^{20}D$ –41.5° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{67}H_{89}O_{15}N_{13}S_2$: C, 58.3; H, 6.50; N, 13.2. Found: C, 58.0; H, 6.52; N, 13.0.

Glycyloxytocin. The protected decapeptide (300 mg) was dissolved in liquid ammonia (500 ml) and sodium was added until a blue color persisted. Ammonium chloride (0.2 g) was then added and the ammonia was allowed to evaporate. The residue was dissolved in water (500 ml) and the solution was aerated at pH 7 until the reaction with sodium nitroprusside was negative, about 4 days being required. Acetic acid (0.5 ml) was added and the solution was pooled with another similar batch. The combined solutions were evaporated *in vacuo* below room temperature to a volume of 30 ml. This solution was placed in the first 5 tubes of a 200-tube countercurrent distribution apparatus. A solvent system of butanol-ethanol-0.05% acetic acid (4:1:5) was used. After 377 transfers the contents of all the tubes except Tubes 80-120 were replaced with fresh solvent and the material was recycled to effect a total of 950 transfers. The plotting of the curve from the Folin color values(9) indicated the presence of one component ($K = 0.36$). The contents of Tubes 14-45 were pooled and 46-80 were pooled, and both fractions were evaporated to small volumes, then lyophilized to give 60 mg and 33 mg of white solid, respectively. On paper chromatography in the system butanol-acetic acid-water (4:1:5) both fractions exhibited a ninhydrin-positive spot with an R_F value of 0.40. A sample of the glycyloxytocin was hydrolyzed in 6 *N* HCl for 16 hours at 110° and subjected to chromatography on the starch column(10). The molar ratios between the constituent amino acids and ammonia were as follows (with the value for leucine plus isoleucine taken arbitrarily as 2): tyrosine 0.9, proline 0.7, glutamic acid 1.0, aspartic acid 1.0, glycine 2.0, cystine 1.0 and ammonia 3.1.

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‡ The melting points reported are corrected, capillary melting points.

Anal. Calcd. for $C_{45}H_{69}O_{13}N_{13}S_2$: C, 50.8; H, 6.54; N, 17.1. Calcd. for $C_{45}H_{69}O_{13}N_{13}S_2 \cdot C_2H_4O_2$: C, 50.2; H, 6.54; N, 16.2. Found: C, 50.2; H, 6.59; N, 16.7.

Summary. 1) The protected decapeptide carbobenzoxyglycyl - S - benzyl - L - cysteinyl - L - tyrosyl - L - isoleucyl - L - glutaminyl - L - asparaginyl - S - benzyl - L - cysteinyl - L - prolyl - L - leucylglycinamide was synthesized by coupling of the *p*-nitrophenyl ester of carbobenzoxyglycine with S-benzyl-L - cysteinyl - L - tyrosyl - L - isoleucyl - L - glutaminyl - L - asparaginyl - S - benzyl - L - cysteinyl - L - prolyl - L - leucylglycinamide. Removal of the protecting groups followed by oxidation to the disulfide gave glycyloxytocin which was isolated by countercurrent distribution. 2) The pharmacological effects of this peptide derivative of oxytocin were investigated by testing it for avian depressor, pressor, and rat uterine-contracting activities. The sample of glycyloxytocin possessed only a slight degree of these 3 activities. It was found to exert a marked inhibitory effect on the avian depressor activity of oxytocin. On the other hand, the glycyloxytocin did not show inhibitory action against oxytocin on the isolated rat uterus or against oxytocin or vasopressin in the assay for pressor activity in the rat. 3) Thus, the attachment of a glycyl residue to the free amino group of oxytocin causes almost complete loss of its avian depressor, rat uterine-contracting, and pressor effects. In addition, it is of considerable interest that this peptide derivative of oxytocin exerts a marked inhibitory action upon the avian depressor effect of the hormone.

The authors wish to thank Joseph Albert for microanalyses, David N. Reifsnyder for assistance in experiments, Dade Tull for some of the biological assays, and Lorraine S. Abrash for analysis of amino acids on the starch column.

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Received June 13, 1960. P.S.E.B.M., 1960, v104.

Release of Histamine-Like Substance from Mast Cells Following Intraperitoneal Administration of Fat Emulsion in Rats.* (25941)

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A disturbing though infrequent reaction to intravenous administration of triglyceride emulsions in patients is a syndrome characterized by skin flushing, dyspnea, sense of chest oppression, cyanosis and lumbar pain(1). These symptoms and signs are frequently associated with rapid rates of infusion and may begin within 1 to 5 minutes; they usually last 5 to 15 minutes and are followed by complete recovery. A subsequent infusion shortly after the reaction has abated or a few days later produces no further untoward reaction of this nature. The anaphylactoid nature of this syndrome suggests that the emulsions may cause release of histamine or other substances affecting smooth muscles. The present study was undertaken to clarify this hypothesis.

Materials and methods. Adult Sprague-Dawley rats were chosen for this study because it has been stated that the peritoneal tissue has abundent number of mast cells with high histamine content(2). Emulsions or solutions used were: (1) a 30% cottonseed oil emulsion‡ in water containing 5% glucose, 0.5% Demal 14, 0.25% Span 20, 0.5% Tween 60, 0.1% sodium cholate, 1% Ethofat C/15, 1% Aldo 28, and 1% purified soybean phosphatides; (2) Lipomul, I.V. (Lot No. 10680),§ a 15% cottonseed oil emulsion containing 1.2% purified phosphatides, 0.3% Pluronic F-68 and 4% glucose; (3) 5% glucose in water, (4) 0.85% NaCl; and (5) 48/80, a histamine liberator.|| Each of the above materials was injected in 20 ml volumes intraperitoneally to animals weighing about 125 g and was left in the peritoneal cavity for 1-1¼ hours. The animals were then decapitated and fluids recovered from the abdominal cavity. The emulsions remained in a highly dispersed state and were examined for their effects on smooth muscles in the preparations described below. Samples of mesentery were fixed in methanol and stained with the Giemsa stain for microscopic examination of mast cells(4). *Preparation for demonstration of bronchiolar constriction.* Guinea pigs weighing about 500 g were pithed and respiration was maintained by air delivered under positive pressure through a tracheal cannula at 20 strokes per minute. A cannula introduced into the pleural cavity was connected to a tambour which recorded excursions of intrapleural pressures on a moving kymograph using the method described by Koessler and Lewis(5). Emulsions or fluids which had been recovered from the rat peritoneal cavity, as well as fresh materials, were injected into the left jugular vein of the guinea pig preparation. Bronchiolar constriction was indicated by sharp diminution in the excursions of the tambour lever. *Preparation for histamine bioassay.* The fluid recovered from the rat peritoneal cavity was assayed for histamine in a muscle bath containing a 2 cm segment of distal guinea ileum in 5 ml magnesium-free Tyrode's solution at 37°C(2). Atropine (10^{-6} M) was added to block spontaneous movements and cholinergic effects. Known amounts of histamine were added for quantitation of responses to the recovered emulsions. Amplitude of ileal contractions was recorded by kymographic tracings.

Results. Bronchiolar constriction. Fig. 1 shows the effect of fresh cottonseed oil emulsion and of emulsion recovered from the rat peritoneal cavity on the guinea pig preparation. The material from the rat peritoneal

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* This work was supported by Medical Research and Development Command, Office of Surgeon General, Dept. of Army, and Upjohn Co., Kalamazoo, Mich.

† Postdoctoral Research Fellow, N.I.H. Present address: Vet. Admin. Hospital, Nashville, Tenn.

‡ Sources and chemical nature of emulsifying agents have been reported(3).

§ Lipomul, I.V., was kindly supplied by Upjohn Co., Kalamazoo, Mich.

|| Kindly supplied by Wellcome Research Labs., Burroughs Wellcome and Co., Tuckahoe, N. Y.

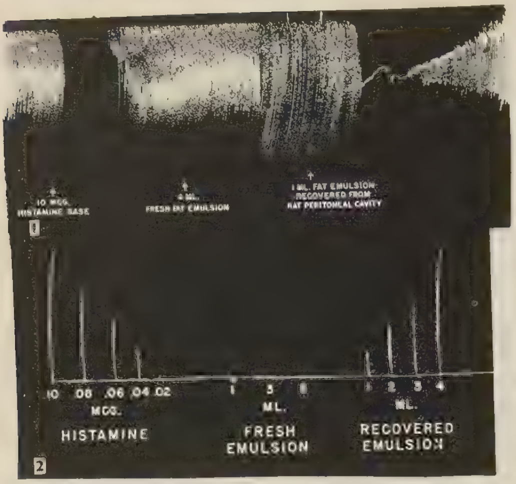

FIG. 1. Effect of fresh cottonseed oil emulsion, emulsion recovered from rat peritoneal cavity and histamine on bronchiolar constriction of guinea pig preparation.

FIG. 2. Effect of fresh cottonseed oil emulsion, emulsion recovered from rat peritoneal cavity and histamine on guinea pig ileal contraction *in vitro*.

cavity invariably caused extreme bronchiolar constriction characteristic of that observed following intravenous injection of histamine. Both the 30% cottonseed oil emulsion prepared in our laboratory and Lipomul, I.V., had this effect. Peritoneal fluid obtained by injection of 0.85% saline or 5% glucose and tested in amounts from 0.1 to 7 ml produced no such effect. When as much as 10 ml of fresh emulsion were infused only minimal respiratory changes were observed. Clustering of the particles of fresh emulsions by addition of bovine albumin also caused no bronchiolar constriction. In fact, whole cottonseed oil injected intravenously in amounts of 0.1 to 5 ml did not produce bronchiolar constriction, although the oil in large amounts was often fatal, presumably due to fat embolization.

Contraction of ileum. The assays for histamine in the peritoneal fluids using the ileal preparation are shown in Fig. 2. Four ml of the recovered emulsions produced contractions of the ileum corresponding to a content of 0.1 μg of histamine base. However, recovered fluids after intraperitoneal administration of 5% glucose or 0.85% saline contained no more than 0.02 μg/ml. The fresh emulsions caused no contraction of the ileal preparations. Addition of 0.1 mg of an antihistamine, Phenergan® (promethazine hydrochloride) to the muscle bath generally blocked the contractions caused by the recovered emulsions, although in occasional experiments this was not complete, suggesting presence of substances other than histamine causing smooth muscle contractions.

Changes in mast cells. Sections of mesentery showed greatly diminished numbers of mast cells after treatment with the emulsions and with 48/80. Many cells appeared to have been disrupted (Fig. 3). The peritoneal stroma did not appear otherwise altered. Normal mast cells were seen after injection of 5% glucose and 0.85% saline.

Discussion. Results indicate that intraperitoneal administration of triglyceride emulsions caused release of histamine, presumably from mast cells of the peritoneum. Since antihistamine did not always completely block activity of recovered emulsions, other vaso active substances such as 5-hydroxy-tryptamine may have also been liberated. These findings suggest that release of agents affecting smooth muscle may play an important role in development of "anaphyloctoid" reactions occasionally observed immediately following initiation of intravenous infusion of fat emulsions in patients. The results clearly indicate that the emulsion itself has no direct action upon ileal smooth muscle *in vitro* nor upon bronchiolar musculature in the pithed guinea pig.

It has been shown that non-ionic detergents of the "Tween" series release histamine when injected intravenously into dogs(6). The pres-

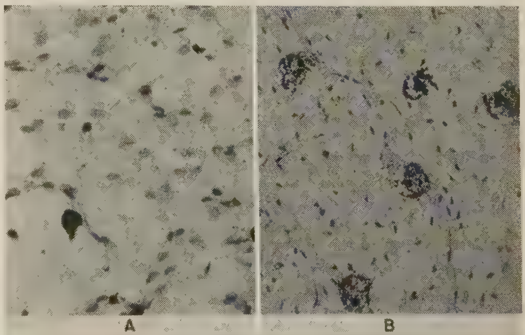

FIG. 3. A. Mesentery of a rat showing normal mast cells. B. Mesentery of a rat 60 min. after intraperitoneal administration of cottonseed oil emulsion showing disrupted mast cells (Giemsa stain).

ent study shows that cottonseed oil emulsion containing other non-ionic detergents are capable of releasing histamine and perhaps 5-hydroxy-tryptamine from tissue cells in rats.

Summary. Two triglyceride emulsions prepared for intravenous use in man have been examined for ability to release histamine from tissue mast cells in rat peritoneal cavity. The emulsions themselves had no direct action upon smooth muscle. Intraperitoneal injection of these materials caused rupture of the mast cells. The fluid recovered from the peritoneal cavity produced bronchiolar constriction in the pithed guinea pig and contraction of guinea pig ileum *in vitro,* indicating a significant histamine content. It is suggested that histamine release may play an important role in development of the occasional "anaphyloctoid" response following intravenous infusion of triglyceride emulsions in patients.

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Received June 13, 1960. P.S.E.B.M., 1960, v104.

Effects of Irradiation *in vitro* on Calcifying Mechanism of Epiphyseal Cartilage.* (25942)

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The calcifying mechanism of vertebrate bone, responsible for deposition of apatite crystals in the organic matrix, has been associated with a specific type of ground substance (1), mucopolysaccharide(2), a specific form of collagen(3,4,5) or collagen in combination with mucopolysaccharide(6). Proteins and polysaccharides have been found to undergo changes when irradiated with x-rays at sufficiently high dose levels(7,8). Thus, it was considered likely that irradiation could be used as a tool for study of the calcifying mechanism. The present investigation is a study of effects of irradiation *in vitro* on calcifiability and metachromatic staining properties of epiphyseal cartilage of rachitic rats. The metachromatic staining was undertaken on fresh sections to observe the system unaltered by histological technics. The effect of irradiation was also studied on rachitic specimens in which the initial phase of calcification had begun. Such sections will be referred to in this paper as nucleated.† Rachitic epiphyseal cartilage was used because it is a convenient system for study of the calcifying mechanism *in vitro* and has been used extensively for studying the intimate mechanism responsible for mineralization(9,10,11). When rachitic epiphyseal cartilage is incubated in serum of normal young animals, or in inorganic solutions containing physiological concentrations of calcium and phosphate, mineralization occurs only in the hypertrophic zone with the same histological distribution as occurs *in vivo.*

Materials and methods. Albino rats, 21 days old, were weaned and maintained for 3 weeks on a modified rachitogenic diet No. 2, U.S.P. The animals were then sacrificed by

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* This investigation supported by contract from U. S. Atomic Energy Comm.

† Nucleated section refers to presence of nuclei of crystallization, which are initial submicroscopic aggregates of Ca and P which can serve as a center about which deposition of bone mineral may occur under appropriate conditions(13).

a blow on the head: Fresh sections including epiphysis and metaphysis, 0.1-0.3 mm thick, were cut free-hand in a frontal plane from proximal part of the tibias. A minimum of 7 intact sections was obtained from the tibias of each animal. Each section was treated by one of the following procedures: (a) Unirradiated section stained with silver by the von Kossa method to demonstrate initial state of calcification at time of sacrifice. (b) Unirradiated section incubated in calcifying solution for 18 hours, then stained with silver, to demonstrate maximum calcifiability under the conditions used. (c) Irradiated sections incubated for 18 hours in calcifying solution, then stained with silver. (d) Unirradiated sections heated at 65° for 15 minutes, incubated in calcifying solution for 18 hours, and stained with silver. This heat treatment results in inactivation of the calcifying mechanism, yet does not interfere with subsequent growth of crystals if nuclei of crystallization are already present(11). (e) Unirradiated section incubated in calcifying solution for 30 minutes to produce nuclei of crystallization, then treated as in (d). This is a control of the nucleation test. (f) Unirradiated and (g) irradiated sections stained with toluidine blue. The *in vitro* calcifying solution contained 9 mg % Ca and 5 mg % P. Its preparation and the technics used for *in vitro* calcification have been described(12). Degree of calcification was assessed by microscopic examination of the silver stained specimens. Sections were irradiated on moistened filter paper placed in glass vials which were set in cylindrical lead shields mounted on a Philips x-ray diffraction unit. The open end of the vial faced the x-ray tube. The radiation used was the unfiltered output of a copper target x-ray tube operated at 40 Kv and 18 ma. Dosage rate at the position of the specimen was 880,000 R/hr as measured with a Victoreen R-meter. While specimens were irradiated, control sections were kept on moist filter paper. The animals were divided into 2 groups depending on whether or not nuclei of crystallization had developed, as demonstrated by procedure (d). Epiphyseal cartilage showed little or no calcification by procedure (a), even when nucleation had occurred. Animals with nucleation usually had a fluctuating weight record. Formation of such nuclei is believed to occur during a period of increased serum inorganic phosphate concentration associated with loss of body weight(13). To study the effect of irradiation on the mucopolysaccharide-protein complex of cartilage, sections (f) and (g) were compared after staining supravitally for 15 minutes in 0.2 ml of a solution containing 0.01% toluidine blue in 0.01 M acetate buffer at pH 3.4. Final pH was 4.4-4.8 due to buffer capacity of the tissue. After staining, the sections were rinsed very briefly in distilled water and examined immediately.

TABLE I. Effect of Irradiation on *In Vitro* Calcifiability of Rachitic Epiphyseal Cartilage.

	Degree of calcification*	
Irradiation	Non-nucleated sections	Nucleated sections
Control	4.0	4.0
100,000 R and below	4.0 (10)	
250,000 R	2.9 (11)	3.2 (10)
500,000 R	1.1 (15)	3.0 (10)
1,000,000 R	.2 (19)	2.3 (10)
2,000,000-4,000,000 R		1.8 (6)

* Figures represent avg for number of sections indicated in parentheses. For each animal, degree of calcification of control sections treated as in (b) was assigned an arbitrary value of 4. Degree of calcification of experimental sections was graded by comparison of area of calcified cartilage with that of control (b), and assigned a value from 0 to 4.

Results. Effect on in vitro calcifiability. The effect of *in vitro* irradiation on calcifiability of rachitic epiphyseal cartilage is summarized in Table I. Preliminary experiments indicated that doses of up to 100,000 R had no detectable effect on calcifiability. In the absence of nuclei of crystallization there was a small decrease of calcifiability following 250,000 R, there was a marked decrease after 500,000 R and almost a complete loss following 1,000,000 R. Calcifiability was affected to a lesser extent in sections which were nucleated before irradiation. After irradiation of 2,000,000 to 4,000,000 R, nucleated sections still retained almost half their ability to calcify.

Effect on toluidine blue staining. In unirradiated sections (f) the small cells of the proliferative zone of epiphyseal cartilage were filled with coarse granules exhibiting β-metachromasia (purple staining), whereas the large cells in matured and hypertrophic zones were filled with fine γ-metachromatic (pink-staining) granules. Capsules of the cells of the proliferative zone were not well stained, whereas capsules of the hypertrophic cells showed a γ-metachromasia. Under the conditions used, staining of the cartilage matrix (except for the capsules) was negligible.

After irradiation with 250,000 R, there was a loss of metachromatic granules from some of the cells of the proliferative region and a small, but more general decrease in staining in the hypertrophic region. After irradiation with 500,000 R, granules were lost from most of the cells of the proliferative zone and from some of the hypertrophic cells. In general, staining was more diffuse, and in some areas the matrix stained. After irradiation with 1,000,000 R, neither cartilage cells nor matrix stained.

These results can be correlated with the color of the residual staining solution after removal of tissue. The toluidine blue solutions used on sections treated with 1,000,000 R were purple whereas those used on sections treated with 500,000 R and 250,000 R were blue with a purple tinge. The toluidine blue solutions used on control sections showed little if any change in color.

Discussion. The calcifying mechanism of rachitic epiphyseal cartilage was inactivated by *in vitro* irradiation at high dosages. There was less inactivation in specimens nucleated before irradiation. Inactivation of the calcifying mechanism by heat or chemical means was reduced or prevented in sections with nuclei of crystallization(11). These results may be interpreted as indicating that a critical phenomenon in calcification is some change in the intercellular matrix capable of inducing formation of submicroscopic nuclei of crystallization. This nucleating mechanism appears to be most sensitive to x-ray, heat, and chemical inactivation. Once nuclei have been formed, the remainder of calcifying process is more resistant to inactivation. The moderate reduction in calcifiability of nucleated sections following irradiation may indicate that the nuclei have some sensitivity to x-irradiation. Another possibility is that the nuclei were not present in sufficient quantity before irradiation to effect complete calcification on subsequent incubation in calcifying solution.

The staining of fresh sections of epiphyseal cartilage with toluidine blue differed markedly from that seen in similar tissues prepared by usual histological methods (*e.g.*, fixation in formalin, Carnoy, or Rossman solutions, followed by embedding in paraffin). With the toluidine blue solution used in our investigation, the matrix of fresh cartilage did not stain, whereas that of fixed cartilage usually stained. Conversely the metachromatic granules were clearly seen in fresh tissues, but were broken up or absent in fixed tissues. The histological treatment resulted in a disruption of the granules and possibly, diffusion of their material into the matrix. In addition, the histological treatment may have freed bound electronegative groups in the matrix which may have been present but masked.

In unirradiated sections (f) stained with toluidine blue, the difference in appearance of the granules in the cells of the proliferative and hypertrophic zones was striking. As the chondrocyte matures and hypertrophies, the relatively large β-metachromatic granules abruptly differentiate into small γ-metachromatic granules.

Metachromasia is presumably due to presence of free electronegative surface charges (14). The change in type of metachromasia of the granules probably reflects an increase in number of free sulfate or carboxyl groups in the mucopolysaccharides, and/or a redistribution of these groups more favorable for metachromasia.

The occurrence of metachromasia in the staining solutions coincident with loss of granules in the irradiated sections suggests that the metachromatic substance was not destroyed by irradiation, but became soluble in the staining solutions. This could be due to breaking of mucopolysaccharide-protein bonds, depolymerization, and increased permeability of the cell membrane.

The effects of irradiation on γ-metachromatic granules and capsules of the calcifiable hypertrophic zone occurred in the same dose range as the effect on calcifiability. This is in contrast to the effects on β-metachromatic granules of the non-calcifiable proliferative zone which were practically complete after lower doses of irradiation that only partly inactivated the calcifiability. The appearance of the γ-metachromasia in the tissue when it becomes calcifiable, and the similar sensitivity of the γ-metachromatic material and calcifiability to irradiation, suggest that a relationship may exist between them.

Summary. 1. X-irradiation of rachitic epiphyseal cartilage with 250,000 R resulted in small decrease in calcifiability, loss of β-metachromatic granules from some cells of the proliferative zone, and a slight general decrease in staining of the hypertrophic zone. X-irradiation with 500,000 R resulted in marked decrease in calcifiability, loss of most of the β-metachromatic granules from the proliferative zone, and loss of γ-metachromatic granules from some cells of the hypertrophic zone. X-irradiation with 1,000,000 R resulted in almost complete loss of both calcifiability and stainability with toluidine blue. 2. Toluidine blue staining of fresh sections of epiphyseal cartilage was employed for demonstration of metachromatic substances. 3. Once the process of calcification had been initiated, the inactivating effect of irradiation on calcifiability was considerably reduced. 4. A change in mucopolysaccharide resulting in increase of free electronegative groups is a factor associated with calcifiability of epiphyseal cartilage.

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Received June 13, 1960. P.S.E.B.M., 1960, v104.

Estrogen Antagonisms: Inhibition of Estrone-Induced Uterine Growth and Vaginal Smear Effects with Testosterone Derivatives. (25943)

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19-Nortestosterone derivatives are highly potent antagonists of uterine growth and vaginal smear effects of estrogens(1). Lengthening of the chain at carbon 17 increased potency in straight-chained forms to ethyl, beyond which potency decreased, and unsaturation affected potency which seemed increased. Data are now available on natural forms of certain of these materials, allowing comparison of structure-activity relationships between 19-nor and natural derivatives of testosterone.

Materials and methods have been described (1). Briefly, in uterine weight studies, various doses of test compound were administered mixed with 0.3 μg of estrone in 0.3 ml of corn oil. 0.1 ml of the mixture was injected daily for 3 days and mice were sacrificed 24 hr after final injection. At autopsy the cleaned, blotted uteri were weighed on a torsion balance. Dose of test compound that was estimated to produce 50% depression in uterine response to the estrone was employed for comparative purposes. Estrone at 0.3 μg generally produces a uterus weighing 35 mg; oil treated mice average 10 mg uterine weight. After 4 days of treatment, vaginal smears were taken from spayed mice on days 5 p.m. and 6 a.m.(2). The presence of leukocytes in fresh smears was the criterion of activity(3). All mice received 2 μg of estrone which generally produces about 90% smears lacking leukocytes, and dose of antagonist estimated to produce 50% depression in this response was employed for comparative purposes.

Results. For both tests progesterone is standard estrogen antagonist and has been assigned a potency of 100%. All 17-substituted testosterones studied were active antagonists (Table I), although their potencies were extremely low. In both tests methyl testosterone was most potent, free testosterone and ethyl being considerably less so. In uterine growth test ethyl testosterone seems somewhat more potent than vinyl or ethynyl, but this is not supported by vaginal smear studies in which ethyl, vinyl and ethynyl are essentially indistinguishable. Results in the two tests show close correspondence of potencies, such as has been found for entire 19-nortestosterone series.

Comparison of results from this test and those from studies with 19-nortestosterones is instructive (Table II). In each case and in both tests 19-nor configuration is considerably more potent than natural. In natural series, methyl testosterone is most potent compound, whereas activities continue to rise to ethyl in 19-nor series. These data indicate that biological effects of modifications of testosterone molecule at carbon 17 are intimately related to presence or absence of angular methyl grouping at carbon 10. This would suggest that both positions are involved in attachment of molecule to whatever receptor site is involved in estrogen antagonisms. This is not a unique finding here, since earlier studies with compounds related to progesterone showed that although neither 6α-methyl progesterone nor 17-acetoxyprogesterone was markedly different in potency from progesterone itself, 6α-methyl-17α-acetoxyprogesterone was almost 3 times more potent than progesterone (3). In the present series free testosterone and ethyl, vinyl and ethynyl testosterone are all of roughly similar potencies, whereas cleavage of the angular methyl grouping at carbon 10 (19-nor configuration) results in major differences in potency among these 4 materials.

TABLE I. Potencies of Various Testosterone Derivatives as Antagonists of Uterine Growth Effects of 0.3 μg of Estrone and Vaginal Smear Effects of 2 μg of Estrone.

	Potency as estrogen antagonist			
	Uterine growth test		Vaginal smear test	
Compound	N*	Potency	N†	Potency
Progesterone	24 (7)	100	141 (4)	100
Testosterone	14 (3)	7	137 (4)	80
Methyl	17 (4)	23	103 (3)	130
Ethyl	11 (2)	14	56 (2)	22
Vinyl	12 (3)	7	57 (2)	27
Ethynyl	11 (3)	8	103 (3)	27

* No. before parentheses indicates No. of groups of 8-10 mice employed in estimation of potency of compound; No. in parentheses indicates No. of tests in which compound was studied.

† No. in front of parentheses indicates total No. of vaginal smears employed in estimation of potency; parenthetical No. is No. of tests in which material was evaluated.

TABLE II. Comparison of Estrogen Antagonistic Potencies of 19-nor and Natural Analogues of Testosterone Derivatives in Mouse Uterine Growth and Vaginal Smear Studies. Progesterone = 100%.

	Potency			
	Uterine growth		Vaginal smear	
Compound	Natural	19-nor*	Natural	19-nor†
Testosterone	7	40	80	170
Methyl	23	880	130	1800
Ethyl	14	1250	22	2100
Vinyl	7	460	27	910
Ethynyl	8	800	27	910

* Data from(1). † In preparation.

Summary. Free testosterone, and its methyl, ethyl, vinyl and ethynyl derivatives were tested as antagonists of estrone-induced uterine growth and vaginal smear changes. Methyl testosterone was most potent of the compounds; there were no major potency differences among remainder. These findings are in contrast to 19-nor derivatives, which are considerably more potent and among which the ethyl is most potent.

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Received June 13, 1960. P.S.E.B.M., 1960, v104.

Estrogen Antagonisms: Effects of Several Steroidal Spirolactones on Estrogen-Induced Uterine Growth in Mice. (25944)

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There has been continuous growth of interest in compounds that antagonize renal action of aldosterone. Pharmacology of the first of these agents to be discovered was summarized by Kagawa, Sturtevant and Van Arman(1), who noted briefly that SC-5233, 3-(3-oxo-17β-hydroxy-4-androsten-17α-yl)propionic acid γ-lactone was inactive as estrone antagonist. Recently, interest has shifted to 19-nor (SC-8109) and 7α-acetylthio (SC-9420) derivatives of SC-5233(2,3). Since Hertz and Tullner(4) were able to demonstrate progestational activity for SC-5233 and SC-8109, it seemed worthwhile to study these materials as estrone antagonists. In addition to these 3 compounds, several other steroidal spirolactones have been studied. Chemistry of these materials has been discussed by Cella and his colleagues(5,6,7).

Materials and methods. Structural formulae for the compounds tested are presented in Fig. 1. Each substance was tested initially at a dose of 1000 μg, against 0.3 μg of estrone, following technic described earlier(8). Briefly, immature mice received test compound mixed with the standard dose of estrone in 3 daily injections, each containing ⅓ of the total doses indicated in this paper. Twenty-four hrs after final injection mice were sacrificed and cleaned uteri weighed on a torsion balance. Compounds were compared at doses estimated to produce a 50% depression in response expected from estrone alone. Earlier experiments, including certain studies dealing with SC-8109, employed Rockland mice and method of evaluation has been discussed(8). Most studies to be described here employed Carworth mice obtained from Small Animals Farms, Des Plaines, Ill., which responded with greater increment of uterine growth. Three groups of 10 animals each that received only estrone averaged 46.3 mg uterine weight; oil-treated controls averaged 9.4 mg. Thus, dose of material estimated to produce uterine weight response of 28.4 mg, which corresponds to a 50% depression, was employed for comparison, and any material that failed to produce this level was considered inactive. In contrast to earlier studies no material considered as inactive here produced any suggestion of inhibition. Progesterone, standard in this test, produces 50% depression at a dose of about 40 μg. Secondary testing of each compound progressed by decreasing dose at increments of about ⅓ until activity was lost. Progestational potencies were determined from subcutaneous Clauberg test(8). For purposes of comparison, potencies were based upon doses that produced a +2 response.

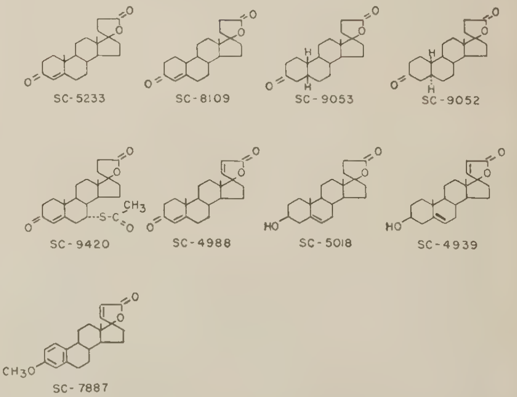

FIG. 1. Structural formulae for various spirolactones tested as estrone antagonists in a mouse uterine growth assay.

TABLE I. Comparison of Estrogen Antagonistic, Progestational and Aldosterone Antagonistic Potencies of a Series of Steroidal Lactones. I = inactive.

Compound	Estrogen antagonist potency	Progestational potency	Aldosterone antagonist*
Progesterone	100	100	1.3
SC-5233	I	100	.22
8109	100	2000	.088
9053	10		>2.4
9052	4		.8
9420	I	I	.33
4988	I	I	>2.4
5018	I	I	”
4939	I	I	”
7887	I		”

* Data from C. M. Kagawa. Figures represent median effective doses.

Results. SC-5233, which might be considered as parent compound in series, was totally inactive as estrone antagonist, confirming Kagawa *et al.*(1), whereas 19-nor-analogue was most potent of series, being about 100% progesterone (Table I). Dihydro-19-nor derivatives were considerably less potent, 5β isomer being about 10% and 5α isomer being approximately 4% progesterone. These structure-activity relationships correlate with those found for relatives of 19-nortestosterone (8) in which 19-nor configuration generally increased potency, and 4,5 dihydro derivatives were less potent than the Δ^4 materials. In dihydro-19-nortestosterone series only isomers with hydrogens at β position at carbons 5 or 10 were active; in present study the 5β material (SC-9053) was more potent than the 5α isomer (SC-9052). 7α-Acetylthio grouping (SC-9420) was accompanied by loss of progestational, but not anti-aldosterone activity. No other structural modification was commensurate with activity.

These studies show no clear correlation between estrogen-antagonistic, progestational or anti-aldosterone activities. SC-5233 has progestational and anti-aldosterone properties, but it is not an estrogen antagonist, whereas its 4,5-dihydro-5β derivative (SC-9053), while maintaining considerable estrogen antagonistic potency, is less than ½ as potent as progesterone as anti-aldosterone. Reverse is true of 7α-acetylthio form (SC-9420) which lacks anti-estrogenic and progestational but retains anti-aldosterone effects. 19-Nor derivative (SC-8109) is relatively potent in all 3 areas.

Summary. A series of steroidal spirolactones, of interest for their aldosterone antagonistic actions, were tested as estrogen antagonists in a mouse uterine growth test. The 2 activities did not appear to be correlated. Progestational activity similarly showed no direct relationships. Structural requirements for estrogen antagonistic potency are discussed. SC-8109 and 2 other 19-nor derivatives of SC-5233, 3-(3-oxo-17β-hydroxy-4-androsten-17α-yl)propionic acid γ-lactone, were estrogen antagonists.

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Received June 13, 1960. P.S.E.B.M., 1960, v104.

Cytotoxic Effect of Leukemic Guinea Pig Plasma on Normal Guinea Pig Spleen Cell Tissue Cultures.* (25945)

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Congdon and Lorenz(1) described occurrence of acute lymphocytic leukemia in an inbred strain (#2) of guinea pigs. The disease was characterized by widespread leukemic involvement of the entire hematopoietic system, with high blast cell counts in the peripheral blood, and could be transmitted by transfer of tumor tissue. We have previously shown that the disease can be reproduced in first generation hybrids, originating from crossmating susceptible strain #2 with resistant Hartley guinea pigs(2), and that transmission succeeds equally well with leukemic plasma or with leukemic spleen extract(3). This paper reports a cytotoxic effect which may be observed when plasma from leukemic guinea pigs is added to normal guinea pig spleen cells growing in primary tissue culture.

Materials and methods. Leukemic plasma was prepared from centrifuged heparinized blood; the blood was drawn from hybrid guinea pigs (strain #2 x Hartley, F_1), inoculated with leukemia L_2C, at height of the disease. Two cycles of cold centrifugation at 5500 rpm for 20 minutes were used to separate plasma from cellular elements of the blood. Plasma prepared by the same method from uninoculated hybrid guinea pigs served as control. Tissue cultures were prepared in square, rubber-stoppered flasks from finely minced, embryonic or adult normal guinea pig spleens (Hartley strain); the cells were suspended in medium 199 which contained 15% normal horse serum, human serum or guinea pig serum (plus antibiotics). Flasks were incubated at 37°C and fluid was replaced thrice weekly with fresh medium. When continuous monolayers of fibroblasts and epithelial-like cells had developed—usually after 12 to 20 days—freshly harvested leukemic plasma was added in a volume of 0.5 ml to 10 ml of tissue culture fluid. The flasks were reincubated and cell sheets were examined microscopically during the next few days for morphological changes. It goes without saying that rigid sterility was maintained throughout all experiments.

Results. Twenty different samples of leukemic plasma were tested as described above. Fourteen samples produced a definite destructive effect which was noted after 24 to 72 hr contact between leukemic plasma and tissue culture cells. This effect began with increased granularity, vacuolization and cutting off of groups of spindle cells; in later stages, this was followed by more or less complete disintegration of both fibroblastic and epithelial-like cells, terminating with exfoliation of the sheet when the effect was maximal (Fig. 1-4). A similar effect was noted in one experiment with leukemic serum and in 3 out of 4 experiments in which twice centrifuged leukemic spleen extract was used as inoculum. Normal guinea pig plasma, or serum, produced no deleterious effects on the cells. Moreover, when leukemic plasma which proved fully active on normal guinea pig spleen cell cultures was added to established cell lines of leukemic guinea pig spleen cell, HeLa cell or human amnion cell cultures or to primary whole embryonic mouse cell cultures, no cytotoxic effects were seen. A certain degree of specificity of the phenomenon is therefore apparent.

The cytotoxic factor in leukemic serum was filtrable through a Millipore membrane of 0.45 μ porosity and was thermostabile up to one half hour heating at 65°C. Titration of leukemic plasma indicated a maximal potency up to a 1:500 dilution. Attempts were made in 5 experiments to maintain the cytotoxic effect over serial tissue culture passages; however, in no case did transfers succeed beyond the quantitative limits of potency of the original inoculum. The results were no different

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* Aided by grants from Leukemia Society, N.Y.C., Am. Cancer Soc., and Nat. Cancer Inst. (PHS grant C-4368).

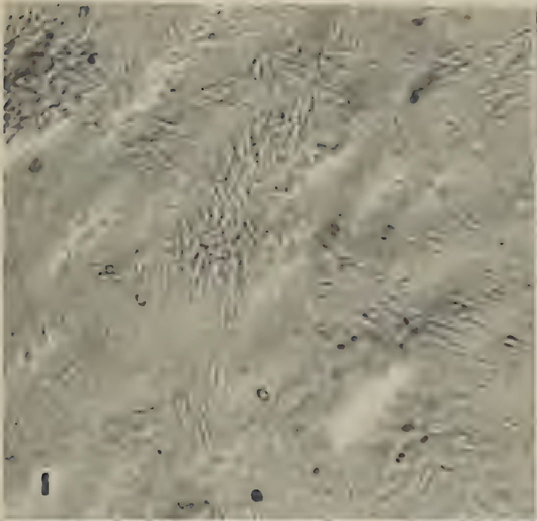
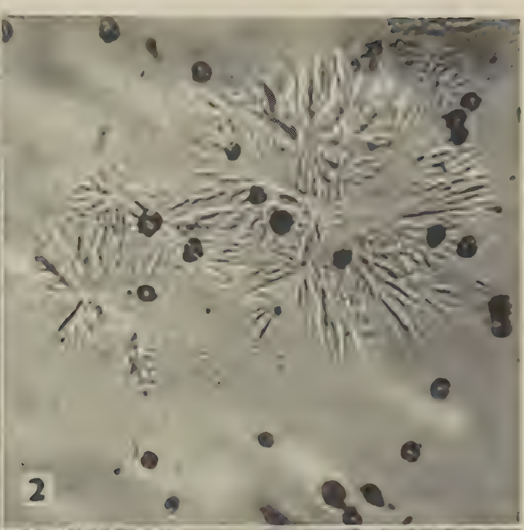
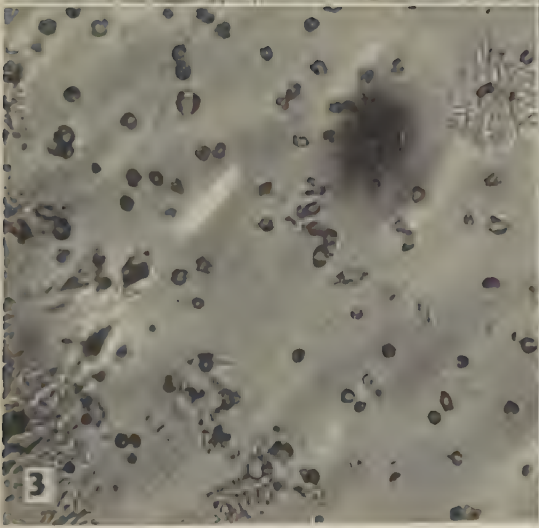
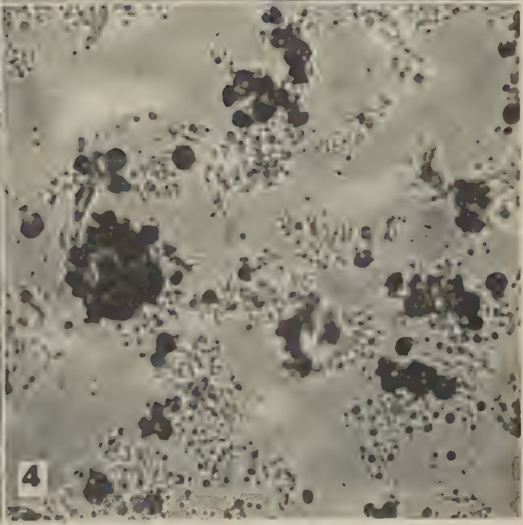

FIG. 1. Normal adult guinea pig spleen cells growing in primary tissue culture (control).
FIG. 2. Early cytotoxic effect produced by leukemic guinea pig plasma.
FIG. 3. More advanced cytotoxic effects produced by leukemic guinea pig plasma.
FIG. 4. Maximum cytotoxic effect produced by leukemic guinea pig plasma.

when tissue cultures were used which had been prepared from strain #2 embryonic guinea pig spleens suspended in strain #2 serum. In 4 different experiments susceptible guinea pigs were injected with tissue culture material representing cells or fluids from flasks with cytotoxicity. None of 6 animals in this group developed clinical signs of leukemia during 3 months observation and none resisted subsequent challenge with leukemic material.

Discussion. It is, of course, generally recognized that certain samples of normal human or animal sera may be toxic for certain cells in tissue culture(4,5). On the other hand, cytopathogenic activity, which is serially transmissible in tissue culture passages, has been observed in controlled experiments in which leukemic material was introduced into suitable tissue culture media. Such effects have been reported by Dmochowski *et al.* (6) with monkey kidney cell cultures inoculated with cell-free extracts from human leukemic lymph nodes and by De Carvalho(7) who inoculated primary human amnion cell cultures with RNA extracted from human leukemic tissues. Further efforts by these authors to identify these cytopathogenic agents by injection of mice with tissue culture material led to inconclusive results with one investigator(6) whereas the other(7) recorded a significant incidence of leukemia in the injected animals. It is against this background of experimental experience by others that our own results appear to be of interest. Judging from our data, there seems to be no question that leukemic guinea pig plasma or spleen extract, both of which are capable of transmitting the disease in susceptible guinea pigs, contain quite frequently a factor which destroys normal cells of the same species in tissue culture. Yet, our work offers no evidence to show that this factor is self-replicating *in vitro* or will induce transformation of normal host cells into malignant cells in tissue culture. The nature of the cytotoxic factor, therefore, remains unknown. It is possible that it represents a nonspecific by-product of the disease, due to some metabolic disturbance, which accumulates in blood and tissues at the height of the leukemic process.

Summary and conclusions. A cytotoxic effect is described which occurs when leukemic guinea pig plasma is added to normal guinea pig spleen cells growing in primary tissue culture. The nature of the cytotoxic factor is unknown.

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Received June 14, 1960. P.S.E.B.M., 1960, v104.

Weight of Adrenal Glands of Cats. (25946)

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Jones(1) states that "A vast amount of information has been collected about organ weights of the laboratory rat, a fair amount for man, the guinea pig and dog, some for the rabbit, cat, mouse and hamster . . ." Since knowledge of the weights of endocrine glands is often of great help in estimating functional status, and data on weights of adrenals of the adult domestic cat are none too plentiful, we are here reporting on a larger series of cats than has heretofore been published. Only 5 papers were found in the literature giving data on weight of cat adrenals, and in only 2 of these is there anything like an adequate series. Latimer(2) reports on 52 males and 52 females, with adrenal weight-body weight ratios of 0.140 and 0.150 g/kg respectively. Butterworth and Mann(3), in connection with another study, give data on 41 males and 48 females, with ratios of 0.131 and 0.148. These authors also report a ratio of 0.111 for 21 castrated males. The other papers have series of 10 or less. Elliott and Tuckett(4) reported on 5 males and 9 females, with ratios of 0.118 and 0.153. Hill(5) gives a ratio of 0.125, based on adrenals of 3 females. Crile and Quiring(6) give a combined ratio of 0.337 for 6 males, 3 females, and one cat of unidentified sex. Surprisingly, the Handbook of Biological Data(7) ignores all previous data and reports a combined average adrenal weight-body weight ratio of 0.2 from 7 males and 3 females.

Methods. The material consists of 85 male

TABLE I. Adrenal Weight-Body Weight Ratios of Cats.

	No. of cats	Body wt, kg			AW/BW, g/kg		
		Mean	95% range	Stand. dev.	Mean	95% range	Stand. dev.
All males	89	2.227	.803-3.651	.712	.147	.047-.247	.050
Small "	17	1.325	1.076-1.574	.124	.172	.087-.258	.043
Large "	15	3.383	2.770-3.995	.306	.124	.080-.167	.022
All females	102	1.886	.955-2.817	.465	.162	.077-.247	.042
Small "	15	1.258	1.026-1.490	.116	.199	.126-.271	.036
Large "	13	2.723	1.985-3.462	.369	.131	.064-.197	.033

and 103 female cats, all adults, which were used in experiments on intestinal absorption. They were killed by heart stab while under barbital anesthesia. The adrenals were removed immediately after death, trimmed, blotted, and weighed to the nearest 5 mg in the fresh state. Body weight of the animals was determined to the nearest 5 g.

Results are summarized in Table I. The groups of cats designated "small" and "large" are those whose body weights fell more than one standard deviation away from the mean. The 95% range is the range from 2 standard deviations below to 2 standard deviations above the mean.

The difference between adrenal weight-body weight ratios of male and female cats of all sizes is significant, by the t test, at the 3% level. The differences between ratios of large and small male cats, and large and small female cats, were both significant at the 0.1% level.

Discussion. The significant differences between adrenal weight-body ratios of males and females, and between those of large and small animals of each sex, are accounted for by the difference in body weight between the groups. If mean adrenal weight-body weight ratio for each group be plotted against mean body weights for the groups, all the points fall very close to a straight line except the value for the small females, which have somewhat larger adrenals than they should have for their body weight. But since the difference between this ratio and that for the small males, of about the same body size, is not statistically significant, this is probably a chance variation.

Brody and Kibler(8) give tables of the expected size of various organs with given body weights, calculated according to the equation $Y = aX^b$, the relative growth equation. Our data do not fit well with theirs, the various groups of cats having average adrenal sizes from 24 to 42% lower than those calculated according to the equation. Christian (9) gives a graph showing the relation between body weight and adrenal weight in various species of animals, not including the cat. Our data fit rather precisely onto his graph.

Summary. Adrenal weight-body weight ratios of 89 male and 102 female cats were determined. The ratio for males is 0.147 g/kg; for females, 0.162. The difference is statistically significant, and is due to a difference in average body weight of the 2 groups. A similar difference in ratios is seen between large and small male and large and small female cats.

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Received June 20, 1960. P.S.E.B.M., 1960, v104.

Attempts to Extract Infective Nucleic Acid of Rous Sarcoma Virus.* (25947)

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Although infectivity of viral nucleic acid (NA) has been demonstrated for 2 plant viruses(1,2,3), several animal viruses(4-13) and T2 bacteriophage(14) the only oncogenic virus for which an infectious NA has been reported is polyoma virus. The original report(16) and a subsequent paper(17) indicate that infective polyoma NA is deoxyribose nucleic acid (DNA) while a recent report suggests that it may be ribose nucleic acid (RNA)(18). Failure to obtain an infective NA from Rous sarcoma virus (RSV) by cold phenol extraction was reported(19). The present study, which also reports failure to isolate infectious NA from RSV is presented because of the variety of technics used, because these negative results suggest that RSV might be fundamentally different from viruses which have yielded infectious NA, because in some experiments non-transmissible cytologic effects were observed which may be of biological significance, and because the studies cannot at this time be continued.

Methods. Two virus lines were used, both originally supplied by Dr. Ray Bryan. "M" line, maintained by both chick wing web (WW) and chick embryo chorioallantoic membrane (CAM) passage, titered about 10^{-5} in both CAM and WW. "B" line, maintained by Bryan's early passage technic(20) titered 10^{-7} or 10^{-8} by CAM or WW. Time of harvest was varied from day 3 to 10 for wing tumors and from day 2 to 7 for infected CAMs and the tissues were frozen immediately thereafter at –70°C (dry ice). Reagents were added directly to the frozen tissue in the homogenizer. The cold phenol technic(1) was used on one CAM and 3 WW preparations of M line virus, and one CAM stock of B line virus. Temperature was approximately 0 to 5°C (ice-water bath). Cadavarine 15 γ/ml was added to one cold phenol preparation to "stabilize" NA(21). Hot phenol technic(9) but with pH 6.7 0.14 M sodium citrate buffer was used on one WW and 9 CAM preparations of B line virus. Temperature was varied between 42° and 50°C. Proportion of the water-phenol interphase which was discarded was also varied. In 2 hot phenol experiments NA was precipitated with 67% ethanol or isopropanol. Sodium deoxycholate (SDC)(15) at final concentrations from 0.03% to 0.67% and with 37°C incubation for periods of 1 to 60 minutes was used in 10 experiments with a single RSV stock prepared from B-line infected CAMs. In 7 of these experiments the SDC was in solution for 2-7 days at 4°C prior to use, but in 3 it was used immediately after preparation. In 3 SDC experiments chymotrypsin 0.5% plus papain 0.5% were incubated with the tumor suspension at 4°C for 4 to 17 hours prior to SDC treatment to inactivate tissue ribonuclease. This did not inactivate virus but the effect on nucleases was not ascertained. In each experiment untreated tumor suspension, the NA preparation, and a ribonuclease-treated aliquot of the NA were tested simultaneously for infectivity by the same technics. Eggs and chickens were white leghorn, from Shamrock Farms, New Brunswick, N. J. NA preparations were tested as undiluted material equivalent to approximately 10% original tissue suspension, in groups of 25 to 40 eggs or chicks. Tissue culture (TC) tests were made in duplicate and usually included 1:5 and 1:25 dilutions as well as undiluted NA. In all but 3 of 25 experiments, CAMs of 9 or 12 day embryonated chicken eggs were inoculated with 0.2 ml of test materials and were examined for pocks on day 19 or 20. Titers on graphs indicate number of CAM-pock-forming units (PFU) per gram of tissue based on serial log dilutions in groups of 5 to 7 eggs. One of the cold-phenol preparations was inoculated

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* Studies supported in part by Phoebe Waterman Fund, Nat. Cancer Inst., U.S.P.H.S., and Atomic Energy Comm.

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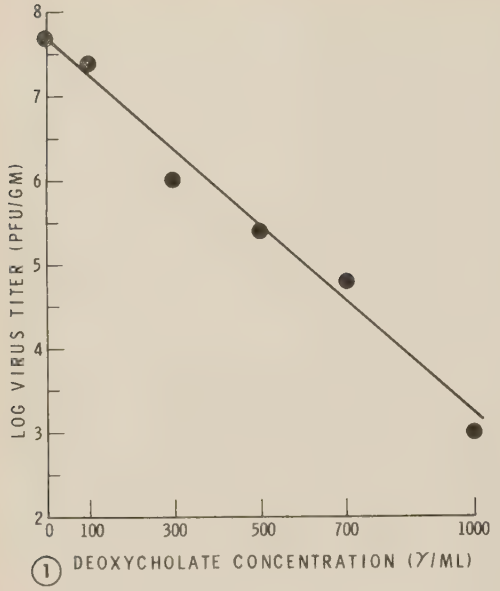
intravenously by Dr. Spencer Munroe into 12-day chick embryos (0.1 ml into veins of the CAM) and 1-day old chicks. Wing web inoculations were used in almost all phenol experiments and half of the SDC studies. Chicks were observed for 6-8 weeks. Hyaluronidase (Alidase, Searle Co.) was used with 2 of the hot phenol preparations at 3 units/ml as suggested by Prince(22). In one cold phenol experiment wings were injected with 0.2 ml of a 2% suspension of diatomaceous earth (Fuller earth powder, Mathewson Co.) 2 hours prior to RNA inoculation at same site, since a previous experiment had indicated that this procedure increased sensitivity of virus detection 10-fold. TCs were monolayers on glass. An agar overlay over chick embryo fibroblasts (23) was used for 10 of the 12 hot phenol preparations through the collaboration of Drs. Maxwell Eidinoff and Arnold Rich. Other TC studies used fluid medium consisting of Gey's salt solution, horse serum, and chick embryo extract. Medium from fluid cultures was routinely passed to CAM and/or WW 7 and 14 days after inoculation. Mengo(24), West Nile(25) and Semliki Forest(26) virus preparations were mouse brain suspensions harvested from paralyzed mice and stored on dry ice. Infectivity was titered by intracerebral (i.c.) inoculations of 16-18 g Swiss white mice (Taconic Farms) with 0.03 ml of serial 10-fold dilutions of NA extracts or brain suspensions and expressed as dilutions causing 50% mortality (LD_{50}).

Results. Cold phenol treatment of Mengo and Semliki Forest viruses and hot phenol treatment of West Nile virus (all as infected adult mouse brain preparations) yielded products with an ultraviolet absorption maximum at 255-260 and infectivity for mice which was abolished by incubation with 25 to 100 γ/ml RNAse for 5-20 minutes at 37°C. Intracerebral LD_{50} titers in 4-6-week-old Swiss White mice for initial virus stocks *vs* NA extracts were: Mengo $10^{-6.5}/10^{-2.5}$; Semliki Forest $10^{-7.2}/10^{-1.7}$; West Nile $>10^{-7.5}/10^{-1.8}$. These results confirm the more detailed studies of others(5,6,12) and indicate that these technics as used in this laboratory did yield an infective viral NA with suitable viruses.

Fifteen attempts to extract infectious nucleic acid from Rous sarcoma virus with cold or hot phenol technics using a variety of virus sources, chemical manipulations and detection systems yielded no tumors or other transmissible lesions. The only phenomenon suggestive of viral activity was the appearance of foci of rounded cells in chick fibroblast cultures(23) with 3 of the 7 hot phenol preparations so studied. These foci resembled those in the whole-virus controls. RNAse treated NA did not yield such lesions. This effect could not be transmitted by passage of tissue cultures (cells scraped from glass and emulsified) into new tissue cultures, eggs, or chicks. In contrast, tissue cultures inoculated with whole RSV so diluted as to yield only 2 foci/plate were passed without difficulty by all these technics. Thus the observed lesions seem to be the result of RNA from the Rous virus preparation (B-line on CAM, titer 10^{-7}), but there is no evidence that they were due to or accompanied by virus propagation. In these 3 experiments NA was tested undiluted and at 1:5 and 1:25 dilutions. At 1:25 numbers of foci per gram of original tissue were 500, 200 and 2 respectively. These numbers were as great or greater than at the lower dilutions, suggesting an inhibitor in the NA preparation.

SDC inactivated RSV rapidly and in direct relation to SDC concentration (Fig. 1). With 0.03% SDC at 0°C a 2-log (99%) inactivation occurred within 1 minute, but thereafter inactivation proceeded much more slowly (Fig. 2). Failure of RNAse to alter this inactivation curve eliminated the possibility that the residual infectivity might be due to RNA. The prompt drop when more SDC was added indicates that leveling-off of inactivation was due to disappearance of effective concentrations of SDC. Much of the SDC was probably inactivated by non-viral constituents of the RSV suspension, since it was shown to be inactivated by prior admixture with bovine serum albumen.

Incubation of one hot-phenol-extracted RSV preparation with an untreated RSV preparation or with RSV partially inactivated by UV irradiation did not affect infectivity titers. Incubation of virus preparations with



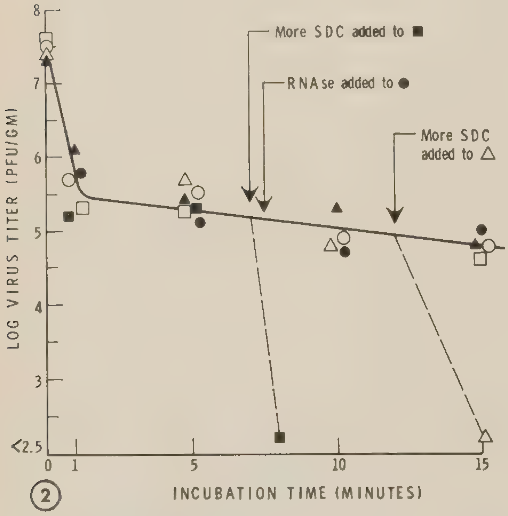


FIG. 1. Decreasing infectivity of RSV in increasing concentrations of SDC at 0-2° (ice water bath) for 5 min.

FIG. 2. Partial inactivation of RSV infectivity by 0.03% SDC at 0-4°C. Infectivity of partially inactivated virus was unaffected by RNase, but abolished by added SDC.

DNAse prior to hot phenol extraction failed to yield an infective NA. These experiments failed to support the working hypotheses that NA preparations might contain inhibitors or incomplete viral components capable of restricted viral activity.

Summary. Sodium deoxycholate and hot and cold phenol were used to extract nucleic acid from Rous sarcoma. The hot phenol technic in some experiments yielded a RNAse-sensitive product that caused non-transmissible focal lesions in chick fibroblast tissue cultures, but no extracts initiated a transmissible infection in tissue cultures, chicks or embryonated eggs. The same hot and cold phenol technics did yield infective nucleic acid from West Nile, Mengo, and Semliki Forest virus preparations. Rous sarcoma virus was rapidly inactivated by deoxycholate.

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Received June 20, 1960. P.S.E.B.M., 1960, v104.

Effect of Triparanol on Beta-Aminopropionitrile-Induced Dissecting Aneurysm and Blood Lipid Levels in the Turkey.* (25948)

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A probable cause of beta-aminopropionitrile (BAPN)-induced dissecting aneurysm in turkeys appears to involve defective connective tissue metabolism, since it has been shown(1, 2) that this is a known effect of the agent. However, the presence of fatty degeneration in the intimal layer of the aorta in the region of rupture in naturally occurring cases(3) of internal bleeding and the gross observation of yellow fatty areas in the lumen of the aorta in the area of rupture in BAPN-treated birds (4,5), raised the question of the importance of atherosclerotic lesions in development of the malady. The object of this study was to determine whether triparanol,[†] a drug having the property of lowering the ratio of blood cholesterol to phospholipid(6), could reduce the severity of BAPN-induced dissecting aneurysm in turkeys. The effects of triparanol on growth rate and plasma cholesterol and phospholipid levels are also reported here.

Methods. Turkey poults were housed during the first 3 weeks in electrically heated batteries with raised wire-mesh floors, then were moved to conventional growing batteries. Details showing breed, number of birds per group, and experimental duration are given in the tables. The diet is presented in Table I. Triparanol and beta-aminopropionitrile fumarate were added as one per cent premixes in ground corn. Vitamin mixture C-5 supplied the following per 5 g of mixture: biotin 0.2 mg, menadione sodium bisulfite—63% USP 2 mg, pyridoxine · HCl 2 mg, folic acid 2 mg, thiamine · HCl 2 mg, riboflavin 5 mg, calcium pantothenate 10 mg, niacin 40 mg, Vit. B_{12}—0.1% triturate in mannitol 30 mg, Cerelose 200 mg, and corn meal to 5 g. All birds succumbing during an experiment and many surviving birds were subjected to necropsy examination. Detailed pathological findings will be published later. Blood samples were drawn in a heparinized syringe as indicated in the tables. Plasma cholesterol and phospholipid were determined by the methods of Abell *et al.*(8) and Zilversmit and Davis(9), respectively.

Results. The depressing action of triparanol on turkey growth is shown in Fig. 1. The depression appears to be logarithmic in nature. The intercept of this straight line relationship with control growth was plotted at 10.6 ppm and 3.9 ppm triparanol for turkeys 5 and 3 weeks on experiment, respectively. It is felt that this is the approximate dosage range at which triparanol begins to depress growth rate in turkeys.

Triparanol did not protect against BAPN-induced aortic rupture (Table II). The hemorrhaging index, as defined by Waibel and Pomeroy(5), was at least as great when triparanol was added to the 0.05% level of

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* Published as paper no. 4343, Scientific Journal Series at Minn. Agri. Expt. Station This investigation was supported in part by research grant from Nat. Heart Inst., P.H.S.

[†] Triparanol was formerly designated by code number MER-29. It is produced by Merrell Co., Cincinnati, O. Formula is 1-[(4-diethylaminoethoxy) phenyl]-1-(p-tolyl)-2-(p-chlorophenyl) ethanol.

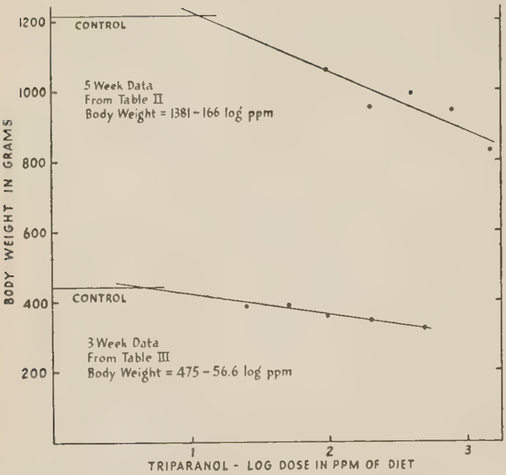

FIG. 1. Effect of graded levels of triparanol on growth of turkey poults.

BAPN. The larger the index, the more severe the expression of internal bleeding, based upon rapidity and degree of incidence. Feeding of triparanol resulted in generally reduced plasma cholesterol levels and increased plasma phospholipid levels, resulting in a markedly reduced cholesterol:phospholipid ratio.

A further experiment was conducted using levels of triparanol ranging from 25-500 ppm (Table III). Growth was depressed even at the lowest level of the drug (Fig. 1). The cholesterol:phospholipid ratio was depressed maximally at lowest level of the drug. However, there appears to be a trend showing decreasing levels of both cholesterol and phospholipid with increasing levels of the drug.

Discussion. According to the intercepts calculated in Fig. 1, it would be expected that triparanol at levels above approximately 10 ppm of diet (approximately 1.0 mg per kg body weight per day) would produce growth depression in young turkeys. Work with rats (10) indicated that 4.1 mg triparanol/kg body weight did not depress growth in a 30 day experiment. This suggests that the turkey is somewhat more sensitive than the rat to low levels of the drug.

Results with rats(10) indicate that triparanol decreased the ratio of blood plasma cholesterol to phospholipid by depressing the former more than the latter. In the present study, the ratio was similarly decreased and the cholesterol level was depressed. However, the phospholipid level was frequently higher in treated birds than in controls.

While growth was depressed even at the lower levels of triparanol, it is assumed that this depression did not influence the blood constituents, since studies utilizing the paired-feeding technic with rats(10) indicated that cholesterol and phospholipid changes were not dependent upon rate of growth.

In previous work from our laboratory(4,5), feeding of BAPN to turkeys until 10 or 21 weeks of age appeared to be responsible for the grossly-observable occurrence of yellow plaques in the area of the abdominal aorta where rupture occurs. In the present study, using turkeys 4 to 6 weeks of age, these lesions were not observable. Preliminary histological evidence with the younger birds indicated that fibrotic intimal plaques were present in the young turkeys, regardless of treatment. These lesions were negative to the fat stains Sudan IV and Oil Red O. Lindsay *et al.*(11) have reported that abdominal plaques in diethylstilbestrol-implanted cockerels were white in younger birds whereas they were yellow in older birds, indicating presence of fat. In view of the non-observance of yellow plaques in these younger birds, it was not possible to fulfill one of the objectives of

TABLE I. Composition of Diet.*

	%
Ground yellow corn	41.65
Soybean meal, dehulled (50% protein)	40.0
Fish meal (60% protein)	6.0
Alfalfa meal (dehydrated, 17% protein)	3.0
Dried whole whey	3.0
Dicalcium phosphate	2.25
Calcium carbonate	2.5
Salt, iodized	.5
Trace mineral mixture†	.1
Vit. mix C-5 (see experimental)	.5
Choline chloride (25%)	.1
Vit. A (5000 I.U./g)	1.1
D (3000 I.C.U./g)	.05
E acetate (44 I.U./g)	.05
DL-methionine	.1
Procaine penicillin G (4 g/lb)	.1

* Diet calculates to contain 28.7% protein and 811 productive energy calories (Fraps, 7) per lb.

† Delamix with 2% zinc, Limestone Products Corp. of America, Newton, N. J., provides diet with the following in mg/kg: Manganese 60, iodine 1.2, iron 20, copper 2.0, zinc 20, and cobalt 0.2.

TABLE II. Effect of Dietary Triparanol on Incidence of BAPN-Induced Aortic Rupture and on Plasma Cholesterol and Phospholipid Levels.*
(Exp. 1)

Level of triparanol (ppm)	At 3 wk				At 5 wk†				
	Wt, g	CHOL, mg %	P-LIP, mg %	C/P	Wt/REM, g	HI	CHOL, mg %	P-LIP, mg %	C/P
No BAPN-F									
0	673	126	266	.477	1211/8	0	129	256	.504
100	600	94	295	.353	1051/7	0	108	295	.386
200	579	108	283	.379	946/6	0	105	285	.369
400	559	88	266	.342	991/8	0	125	352	.354
800	628	103	278	.302	935/8	0	133	377	.352
1600	541	96	272	.352	814/8	0	135	373	.363
.050% BAPN-F									
0	521	127	211	.502	736/3	8.0			
100	600	107	245	.437	946/1	20.0			
200	583	110	270	.408	—/0	22.5			
400	525	107	240	.451	865/3	6.7			

* Experiment started at 7 days of age with 10 Broad Breasted Bronze males/group after pre-experimental period during which poults received control ration. Avg wt was 114 g. CHOL indicates blood plasma cholesterol level. P-LIP indicates blood plasma phospholipid level. Blood values represent avg of duplicate pooled samples of 3 poults each. C/P is cholesterol-phospholipid ratio.

† REM indicates remaining birds at 5 wk. Some were removed at 4 wk for tissue study. Otherwise, non-hemorrhaging mortality in experiment was negligible. HI = Hemorrhage index: 0 equals no aortic hemorrhage, higher numbers indicate increasing severity.

the present study; namely, the effect of triparanol on development of BAPN-induced yellow plaques.

Estrogens are noted for their effects on cholesterol:phospholipid ratio(12). As triparanol may be considered as a substituted stilbene, and therefore potentially estrogenic in action, the effect of triparanol on oviduct weight was studied. Samples of females fed triparanol at 200 ppm were sacrificed after 47 and 61 days on the drug. Mean oviduct weights, whether calculated from absolute values or percentages of body weight, were depressed. Testis weights on males similarly treated showed a weight depression, which was probably not due to estrogen-like inhibition of gonadotropic secretion but due to the general toxic effect of the drug on growth.

A striking observation in this work was that the liver of many triparanol-fed birds exhibited greenish discoloration. It is possible that the color is an accumulation of a decomposition product of triparanol.

Summary. Addition of 25-1600 ppm triparanol to the diet of turkey poults lowered the ratio of plasma cholesterol to phospholipid. Incidence of beta-aminopropionitrile-induced dissecting aneurysms was not lessened

TABLE III. Effect of Triparanol on Growth and Blood Lipids after 3 Weeks on Experiment.*
(Exp. 2)

Level of triparanol (ppm)	Avg body wt, g	Gain / Feed	Blood plasma		
			Cholesterol, mg %	Phospholipid, mg %	Ratio
0	441	.53	100	164	.62
25	388	.48	86	187	.47
50	382	.46	76	164	.47
100	356	.46	77	187	.42
200	346	.47	62	146	.44
500	320	.49	67	154	.47

* Newly hatched poults were placed on 4-day standardization period on control diet, prior to equalized segregation into experimental lots. Broad Breasted Bronze and Broad Breasted White turkeys were housed in separate pens. Each group contained 5 males and 5 females. Avg values of breed and sex are presented. Each plasma value is avg of 16 individual samples, acquired equally from breeds and sexes.

by triparanol. Degree of growth depression due to triparanol was proportional to logarithm of dose.

We express appreciation to Dr. T. R. Blohm of Merrell Co., Cincinnati, O., for supply of triparanol and to Dr. D. V. Frost, Abbott Labs., No. Chicago, Ill. for beta-aminopropionitrile fumarate.

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Received March 14, 1960. P.S.E.B.M., 1960, v104.

Prevention of Malignant Cell Growth and Spread by Tropical Pretreatment with Radioactive Chromic Phosphate.* (25949)

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We have reported(1) that intraperitoneal pretreatment with radioactive Yttrium (Y^{90}) compounds prevented growth and implantation of malignant cells not only by acting directly on tumor cells, but also by increasing indirectly the resistance of the cavitary wall to their invasion. This line of research was continued in our recent experiments, using as the radioactive agent an isotope with a longer halflife and, as targets, tumor inocula in different locations. The results are reported below.

Material and methods. (A) *Mice and tumor strain.* Swiss Albino mice (Albino Farms, Red Bank, N. J.) and CFW mice (Carworth Farms, New City, N. Y.) of either sex, 24 to 27 g weight, were used. Krebs-2 tumor was maintained in serial transfers as "ascites tumor." (B) *Tumor cell inoculation and pretreatment with isotope.* Methods of inoculation of requisite number of tumor cells, of ascitic fluid withdrawal and of the work with isotopes have been described(2,3,4,9,10). *Radioactive chromic phosphate* in colloidal suspension was obtained from Abbott Labs, Oak Ridge, Tenn. This compound of a pure beta emitting phosphorus P^{32} is widely used as a substitute for colloidal radiogold (Au^{198}) which is a beta and gamma emitter(5,6,7,8, 11,12). (C) *Technic of injections.* (aa) *Intrapleurally.* Puncture of most areas of the thoracic wall will produce hemorrhages from the surfaces of the lung. However, it is quite safe to puncture the phrenico-costal sinuses (which are not occupied by the lung), in the area of the right thoracic wall at intersection of posterior axillary line and lowest intercostal space. This procedure was done. (bb) *Subcutaneously into scalp.* We have reported(4) sharply localized growth of scalp tumors in proportion to size of inoculum and their suitability for estimation of normal tissue invasion by tumor outgrowth. For either tumor inoculation or isotope injection a gauge 23 needle was introduced through the external ear into subcutaneous tissue of neck, scalp, nasal region and supraorbital region (see be-

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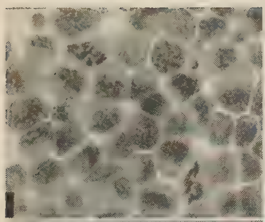
* This work was supported by grants from Division of Biology and Med., U. S. Atomic Energy Commission, and from the Damon Runyon Fund.

low "Pattern of Experiments"). (cc) *Subcutaneously* into abdominal wall and (dd) *intramuscularly* into leg. Routine technics were used, but the isotope was injected into the selected area in 10 fractions of the requisite dose, diluted for this purpose to the volume of 2.0 or 3.0 ml. After skin (and muscle) puncture the direction of the needle was changed after each injection of 0.2 or 0.3 ml of diluted dose until 10 fractions of the dose had been infiltrated into the selected area at each of 10 different points. For intrapleural pretreatment dose of the isotope was injected only once, in a volume of 0.5 ml. Tumor inoculation after pretreatment was given in a single injection in all series. (D) *Safety of pretreatment method.* We have assayed by various routes increasing doses (.1 to .3 mc) of $CrP^{32}O_4$ for their systemic (lethal) and local (in normal tissue of injected area) toxicity in mice. (E) *Scale of quantitative estimate of tumor growth.* (aa) *Amount of pleural exudate* was estimated as proportionate to volume of fluid obtained by insertion of a glass capillary through the thoracic wall: + = 0.2 ml or less; ++ = 0.2 to 0.5 ml; +++ = more than 0.5 ml. (bb) *Invasion of pleural fluid by free tumor cells.* Technic of tumor cell counts in the fluid has been described(2,3,4). According to our experience, decrease in number of tumor cells per cu. mm (4,9,10) and/or in their percentage in the cellular exudate(9,10) reflected the effect of injected radioisotope. (cc) *Extent of intrapleural implantation*: 0 = no implant; + = implant either in superior or inferior mediastinal pleura; ++ = implants in both areas; +++ = spread of mediastinal pleura implant on diaphragmatic or costal pleura. (dd) *Extent of tumor spread in scalp*: 0 = no growth; + = circular elevation on the top of scalp reaching eventually orbits, but without involvement of eyelids; ++ = swelling covering the whole area between neck and orbits (including eyelids); +++ = growth extension on face and neck; ++++ = extension on the back. (ee) *Subcutaneous and intramuscular implants*: 0 = no implants; + = diffuse induration at site of inoculation; ++ = nodule 0.2 to 0.5 mm in diameter; +++ = tumor larger than 0.5 mm in diameter. (F) *Pattern of experiments.* A single dose of 0.1, 0.15, or 0.2 mc of $CrP^{32}O_4$ was given either in a single injection in a volume of 0.5 ml (intracavitary) or infiltrated in a volume of 2 to 3 ml in 10 fractions, each of 0.2 to 0.3 ml, (subcutaneously or intramuscularly) into a selected tissue area. One, 3, or 5 days later an inoculum of 10^6 to 10^7 tumor cells was introduced into pretreated cavity or tissue area. In intrapleural experiments, fluid was withdrawn from the cavity for tumor cell counts on 4th day after inoculation; 2 days later the mice were sacrificed for autopsies and recording of implants; in 5 pretreated animals of each series the withdrawn fluid was inoculated intraperitoneally into new mice for assay of tumor cell viability(3); smears were prepared from all fluid specimens (undiluted) and stained with Acetoorcein(2). In scalp experiments, size and extension of tumor were recorded on 10th day; in abdomen and leg experiments, on 7th day. Recorded data were tabulated and are presented below.

Results. A. *Safety controls.* All new mice survived doses of .2 mc intrapleurally and 0.25 mc subcutaneously and intramuscularly without any macroscopic damage to normal tissues. Higher doses induced occasionally local damage (pleural hemorrhages, crusting and loss of hair in the skin). Dose of 0.3 mc was lethal for all mice. Accordingly, doses of .15 mc and .2 mc injected for pretreatment into new mice were well tolerated. B. *Pleural cavity.* In mice inoculated intrapleurally 1 to 3 days after topical pretreatment, initial proliferation of free tumor cells was arrested, their mitoses disappeared, and their cytoplasm became swollen, thus enlarging their size and leading to their final disintegration (Fig. 2 as compared with controls in Fig. 1). The remaining cells were scanty and non-viable as it appeared from their inability to initiate intraperitoneal growth after transfer into new mice (bioassay of viability, see ref. 4). Their ability to implant into the pleura or to grow there after early implantation was considerably impaired (Fig. 4 as compared with Fig. 3). Table I shows the relationship between extent of these effects, on the one hand, and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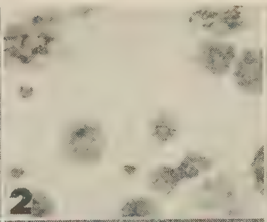
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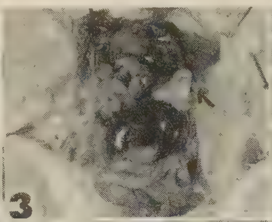
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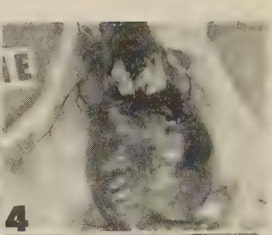
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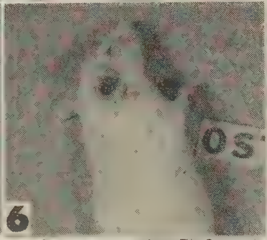

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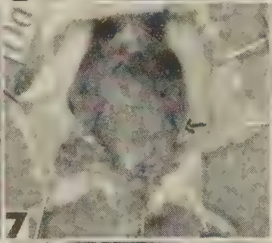
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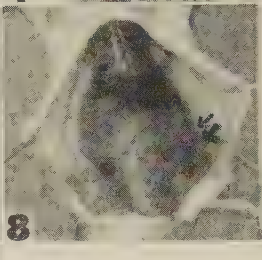
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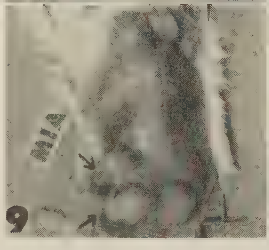

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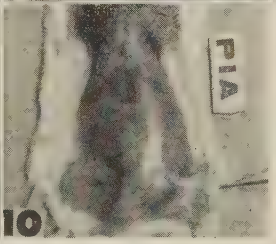

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FIG. 1 and 2. Krebs-2 tumor cells in undiluted pleural exudate of Swiss Albino mice on 4th day after inoculation of 10^6 cells. Smears from 0.2 mm^3 of undiluted fluid. Fig. 1, control: numerous typical tumor cells and their mitoses. Fig. 2, pretreated intrapleurally with 0.15 mc $CrP^{32}O_4$: scarce, distorted tumor cells, macrophages and leukocytes.

FIG. 3 and 4. Pleural implants on the 5th day after inoculation (as in mice 1 and 2). Fig. 3, control: large tumor on diaphragm. Fig. 4, pretreated: no visible tumor growth.

FIG. 5 and 6. Krebs-2 implant in the scalp, 10 days after inoculation of 10^7 cells. Fig. 5, control: whole scalp thickened by tumor growth spreading on neck. Fig. 6, pretreated with 0.15 mc of $CrP^{32}O_4$ into the scalp: tiny necrotic nodule on top of normal looking scalp.

FIG. 7 and 8. Tumor in mouse 6 days after inoculation into skin of abdomen. Fig. 7, control: diffuse spread of tumor tissue covering peritoneum of low abdomen. Fig. 8, pretreated in the same skin area: few tiny necrotic nodules.

FIG. 9 and 10. Tumor 6 days after intramusc. inj. into thigh. Fig. 9, control: large round tumor masses infiltrating thigh and dorsal muscles. Fig. 10, pretreated in the same muscle: small necrotic nodule in right thigh muscle.

dose of radioisotope and interval between pretreatment and inoculation, on the other hand.

It appears from Table I that pretreatment with a higher dose (0.2 mc) shortly (1 to 3 days) before inoculation inhibited completely (or almost so) fluid accumulation and proliferation of free tumor cells and their implantation, while a lower dose (0.15 mc) or an earlier pretreatment (5 days before inoculation) achieved similar effects generally at a lower level and in smaller number of animals. Still, even in series 7 (0.15 mc, interval of 5 days) the results in some mice were close to findings in series 2 (0.2 mc, 3 days). High effect was obtained occasionally even with a still lower dose (0.1 mc). The significance of these variations will be discussed below.

B. *Subcutaneous and intramuscular growth.* Pretreatment with either 0.15 mc or 0.2 mc produced about the same level of tumor inhibition: effects of lower doses (0.1 mc) and of earlier pretreatment (5 days before inoculation) varied widely. Data of experiments in 3 different body areas are reviewed in Table II. According to Table II, standard procedures of pretreatment inhibited practically completely tumor growth in the scalp from standard inocula (Fig. 6). In pretreated areas of the abdominal subcutaneous tissue and of leg muscle, only a small amount of growth, mostly necrotic, was recorded (Fig.

TABLE I. Inhibition of Free Malignant Cell Growth in Pleural Cavity by Intracavitary Pretreatment with Radioactive Chromic Phosphate, in 8 Series.

Schedule of inoculation and treatment		Findings in pleural cavity (avg / variation extremes)			
Dose of inj. radioisotope (mc)	Days between pretreatment and inoc.	Approx. amt of pleural fluid	% of tumor cells in the fluid	No. (thousands) of tumor cells/mm³	Amt of implantation
.2	1	0 / 0 - +	3.5 / 1.7- 5.1	18.8 / 6.1-24.2	0
.2	3	" / "	10.4 / 6.5- 14.2	28.2 / 16.3-44.4	0
.2	5	+ / + - ++	12.2 / 7.1- 16.6	48.8 / 31.5-57.5	+ / 0 - +
Untreated control		++ / ++ - +++	97.4 / 91.0- 98.0	68.2 / 53.1-78.0	++ / ++ - +++
.15	1	0 / 0 - +	8.1 / 4.6- 17.1	21.6 / 7.7-29.2	0 / 0 - +
"	3	+ / + - ++	9.4 / 6.7- 19.9	34.4 / 16.6-41.3	+ / 0 - ++
"	5	" / "	24.2 / 15.5- 31.3	44.9 / 28.0-55.1	" / "
Untreated control		++ / ++ - +++	96.4 / 93.2-100.0	63.2 / 52.0-72.1	++ / ++ - +++

Twenty mice in each series. Size of inoculum = 10^6 tumor cells.

Standards for estimate of fluid volume and implantation extent see *Material and methods.*

In series 1 and 5 tumor cells, mostly atypical, were counted only in mice containing sizable amount of fluid.

Non-neoplastic cells in fluid: granulocytes and small lymphocytes in controls; macrophages and large lymphocytes in pretreated mice.

8, 10) in the majority, and absence of any growth in a sizable number of pretreated mice. In each localization, extent of inhibition was at its maximum in animals pretreated a day before inoculation.

Sections from mediastinal pleura (serosa and subserosa), of the scalp, and of the leg muscle showed in pretreated mice edema of tissue and clumping of tumor cells within pretreated and inoculated area and their absence beyond its periphery, while in controls numerous proliferating cells and islands of organized tumor tissue were found scattered widely far beyond site of inoculation. The role of these phenomena in tumor inhibition by pretreatment will be discussed below.

TABLE II. Inhibition of Malignant Growth and Spread of Subcutaneous and Intramuscular Inocula by Topical Pretreatment with Radioactive Chromic Phosphate (0.15 mc) in 9 Series.

Area of pretreatment and of tumor inoculation	Days between pretreatment and inoc.	Extent of tumor growth and spread on 8th day (avg / variation extremes)
Scalp	1	0 / 0 - +
"	3	" / "
Untreated controls		++ / + - ++
Skin of abdomen	1	+ / + - +++
Idem	3	" / "
Untreated controls		++ / ++ - +++
Left leg	1	+ / 0 - +
Idem	3	+ / + - ++
Untreated controls		+++ / ++ - +++

20 mice in each series.

Doses of 10^6 to 10^7 Krebs-2 ascitic cells were inoculated.

Scale of estimate: See *Material and methods.*

Doses of 0.15 mc of $CrP^{32}O_4$ were used in all experiments.

Discussion. In our previous experiments (1) on growth inhibition of ascites tumors by intraperitoneal pretreatment with a very short-living isotope (Y^{90}, half-life 2.7 days), maximum effect was found in mice inoculated 3 days after pretreatment, *i.e.*, at the time when stored radioactivity had considerably decayed. These findings and microscopic evidence of inhibited tumor cell spread in pretreated tissue suggested that a decrease of tissue susceptibility (or, in other words, increase of its resistance) to cell invasion, rather than irradiation of tumor cells was responsible for the effect of pretreatment with Y^{90}. In our investigations reported here, a longer living isotope (P^{32}, half-life 14.7 days) was used, and it is understandable that amount of radioactivity in the pretreated tissue at time of inoculation was the major factor of tumor inhibition. Indeed, in averages of all experiments presented in Tables I and II, this inhibition was directly proportionate to doses of the isotope and inversely to interval between its injection and tumor inoculation. However, findings in sections of pleura and subcutaneous tissue pretreated with P^{32} were analogous to previously described changes in the peritoneum pretreated with Y^{90}. Accordingly, they were given similar interpretation. It may be presumed that irradiation with P^{32} decreased viability in tumor cells, and in the pretreated tissue, its susceptibility to invasion by these cells (or, in other terms, increased its resistance). This interpretation is in agreement with the experience of Andrews, Root and Kniseley(11) that "some unknown mechanism in action, possibly on surfaces not involved with tumor, may be important" for therapeutic action of Au^{198} in human peritoneal carcinosis.

Wide range in variations of results was found in series 7, Table I, and series 8, Table II, where radioactivity level in tissue was expected to be at its lowest. In these conditions, extent of tumor cell inhibition depended more considerably on tissue response to irradiation, which probably varied individually within the same series of mice.

In the same conditions of experiment, extent of inhibition by pretreatment was highest in scalp and lowest in subcutaneous tissue of the abdomen. These differences suggest the importance of tissue characteristics (loose or dense connective tissue, vascularization, etc.) and of its topographic localization in the mechanism of tumor prevention by pretreatment. Radioactivity doses of .15 mc achieved considerable preventive effect by all routes. This dose was found to be well below the maximum tolerated doses (.2 mc intrapleurally and .25 mc by other routes) and moreover below doses required for therapeutic use(7,8,12) in tumor-bearing mice. Accordingly, preventive use of the isotope offers advantages of greater safety than its therapeutic use.

Summary and conclusions. (1) Pretreatment of new mice with $CrP^{32}O_4$ in well tolerated doses for topical prevention of tumor growth and spread was applied in various topographic areas, *i.e.,* pleural cavity, scalp, abdominal wall and leg. (2) Reduction of tumor growth in all areas and of its spread in pleural and scalp were directly proportional to dose of radioactivity and inversely to the period between isotope injection and tumor inoculation. (3) These findings showed that amount of radioactivity stored in pretreated tissues at time of tumor inoculation was the major factor of the isotope's preventive effect. However, previous results(1) suggested also another effect of pretreatment, that of tissues increased resistance to invasion with tumor cells. (4) It was concluded that topical pretreatment with $CrP^{32}O_4$ may be attempted for prevention of postoperative growth and spread of tumor cells.

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Received April 5, 1960. P.S.E.B.M., 1960, v104.

Effect of Raw Soybean Meal and Amino Acids on Pancreatic Hypertrophy in Rats. (25950)

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A number of physiologically active constituents have been reported in raw soybean meal, including a pancreas stimulating factor (1), trypsin inhibitor(2), goitrogenic substance(3), saponins(4), estrogenic substances (5), hemagglutinin(6), and anticlotting factor(7). However, when we fed raw soybean meal to rats as the sole source of dietary protein (14%), the only adverse effects noted were (a) poor growth, (b) reduced food efficiency and (c) pancreatic hypertrophy. Borchers(8) reported that growth inhibition in rats due to raw soybean meal can be counteracted by addition of 4 specific amino acids to the diet. In the present study enlargement of the pancreas was observed when rats were fed amino acid-supplemented raw soybean meal diet.

Methods. Weanling male albino rats from our colony were separated into 4 uniform groups of 5 according to weight. Animals were housed in individual wire-bottom cages and fed diets *ad lib.* Group 1 received control (casein) diet which contained in per cent: cerelose 50.7, cornstarch 20, crude casein 17.3 (N x 6.25 = 14.0% protein), salts (USP XIV) 4, complete commercial vitamin mix 2, and powdered cellulose 2. Group 2 received above diet with 28.4% raw soybean meal (N x 6.25 = 14.0% protein) substituted for casein (17.3%), cerelose (9.1%), and powdered cellulose (2.0%). Group 3 received the raw soybean meal diet plus 4 amino acids (0.6% L-tyrosine, 0.6% DL-methionine, 0.6% DL-threonine, and 0.2% DL-valine) substituted for equal amount of cerelose. Group 4 received 28.4% heated soybean meal (autoclaved at atmos. pressure for 30 min). Weekly records of body weights and food intakes were maintained. After 36 days rats were sacrificed and pancreatic tissue including extraneous fat was carefully excised, preserved in 10% aqueous formaldehyde for 48 hours, then carefully trimmed, blotted and weighed. Food efficiency was calculated for each rat by dividing grams gain in body weight by grams of food consumed during the 36-day period. Since all groups received the same dietary level of protein (14%), it follows that food efficiency and protein efficiency of utilization are directly related.

Results. The data in Table I show that significant growth inhibition, increased pancreatic weight and reduced food efficiency resulted when raw soybean meal was substituted for casein. When 4 amino acids were fed with raw soybean meal (Group 3), growth and food efficiency were no longer significantly different from the control group. However, hypertrophy of the pancreas was not prevented by amino acid supplementation. No adverse effects were observed in Group 4 receiving heated soybean meal.

The following tissues were examined microscopically: heart, lungs, thyroid, testes, spleen, liver, pancreas, kidney, adrenals and intestine. All organs were normal except the pancreases of animals fed raw soybean meal with or without amino acids, (Fig. 1 and 2). Pancreatic acini had lost their regular circular outlines and were jumbled and without distinct lumens. The basilar portion of the hyperplastic acinar epithelium was intensely basophilic and increased at the expense of the zymogenic portion. Ribonuclease digestion studies indicated this basophilic cytoplasm to consist predominately of ribose nucleic acid. Mitoses were occasionally encountered. Acini still spherical enough to be measured were significantly smaller ($P = <0.01$) than normal controls, apparently due to squeezing by adjacent hyperplastic cells. Nucleoli were significantly larger ($P = <0.05$); nuclei appeared larger but were not statistically so. Pancreatic islets appeared unaffected.

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* A laboratory of Western Utilization Research and Development Division, Agri. Research Service, U. S. Dept. of Agriculture.

TABLE I. Effects of Raw and Heated Soybean Meal and Amino Acids on Growth, Pancreas Weights and Food Efficiency of Rats.

Dietary protein	Final body wt, g*	Pancreas wt, g/100 g body wt*	Food efficiency, wt gain/g food*
(1) Casein	147.40 ± 3.88	.56 ± .04	.31 ± .01
(2) Raw soybean meal	89.00 ± 3.54†	.85 ± .08†	.19 ± .007†
(3) *Idem* + amino acids	142.40 ± 7.11	.93 ± .06†	.28 ± .01
(4) Heated soybean meal	148.40 ± 3.27	.50 ± .02	.29 ± .006

* ± S. E. † P = <0.01.

Discussion. The original observation of pancreatic hypertrophy in chicks fed raw soybean meal(1) is now extended to include the rat. The observation of Borchers(8) that growth inhibition may be reversed by addition of tyrosine, methionine, threonine and valine to a raw soybean meal diet is confirmed. Since added amino acids did not prevent pancreatic hypertrophy, poor growth is obviously not the cause of pancreas enlargement. According to Lyman and Lepkovsky(9) and Lyman(10), excessive amounts of pancreatic enzymes are emptied into the intestine and excreted in the feces when either raw soybean meal or soybean trypsin inhibitor concentrate is fed to rats. These workers concluded that growth depression may be exerted through a loss of essential amino acids from endogenous sources rather than through depression of normal intestinal proteolysis. Support for the concept of fecal endogenous amino acid loss, rather than incomplete intestinal proteolysis, is the finding that decreased growth rate and protein utilization resulted when soybean trypsin inhibitor concentrate was fed with hydrolyzed casein to mice(11). Other workers(12,13) have reported that when 2 dietary levels of raw soybean meal are compared for inhibition of chick growth, the high level gives better growth than the low level. If it is assumed that maximal pancreatic stimulation occurs at the low level, then it is not surprising that the high level of raw soybean meal would support better growth by increasing dietary supply of amino acids to overcome endogenous loss of amino acids *via* the feces. Sources and levels of dietary nitrogen used may be the key to some of the conflicting conclusions that have been reported regarding toxicity of soybean fractions.

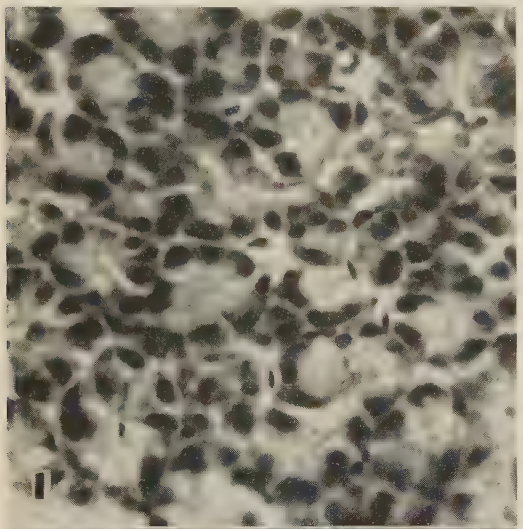

FIG. 1. Normal rat pancreas. Hematoxylin and eosin stain. ×540.

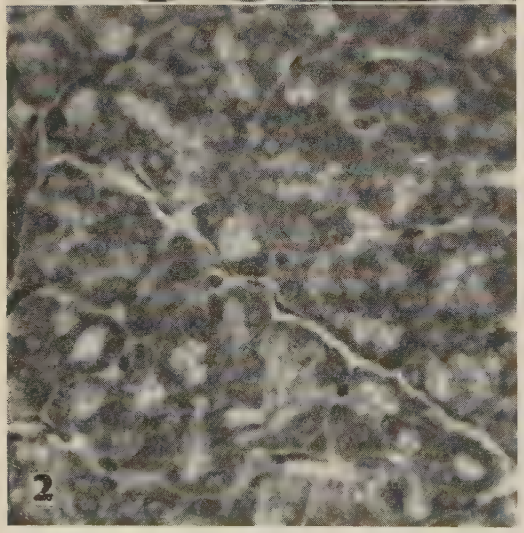

FIG. 2. Pancreas of rats fed 25% raw soybean meal for 22 days. Hematoxylin and eosin stain. ×540.

Summary. Pancreatic hypertrophy, poor growth and lowered food efficiency result from feeding raw soybean meal to rats as sole source of dietary protein. Addition of 4 specific amino acids to raw soybean diet corrects poor growth and reduced food efficiency but does not prevent pancreatic hypertrophy. These results support the concept that decreased growth rate and protein efficiency caused by feeding raw soybean meal are due to direct stimulation of the pancreas resulting in excessive loss of critical amino acids contained in pancreatic enzymes excreted in feces.

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Received April 14, 1960. P.S.E.B.M., 1960, v104.

A Latex Agglutination Test for Anaerobic Diphtheroids. (25951)

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Serological observations with anaerobic diphtheroids have been limited because of the tendency of these organisms to grow in clumps and to agglutinate spontaneously. This communication is to report a latex (polystyrene) agglutination test for anaerobic diphtheroids which is reproducible, easy to read and not influenced by spontaneous bacterial agglutination.

Materials and methods. Organisms were characterized as anaerobic diphtheroids if they required an anaerobic environment for multiplication, had a pleomorphic rod-like or branching appearance, were Gram positive, produced catalase and did not ferment xylose, salicin or raffinose(1). All of our anaerobic diphtheroid strains were recovered and maintained in fluid thioglycollate medium. Most of the other bacteria mentioned in the text were obtained from the American Type Culture Collection and maintained in appropriate media. *The latex test* required an antigen-latex-buffer mixture which was prepared by mixing a suspension of washed bacteria (density to match McFarland #8 standard), 0.81 μ latex particles and glycine saline buffer (pH 8.2). (One part of the stock suspension of latex was diluted with 4 parts of distilled water to give the proper concentration of latex.) To prepare 5 ml of this mixture: 0.5 ml of bacterial suspension, 0.2 ml of diluted latex and 4.3 ml of buffer were mixed and allowed to stand at room temperature for at least 10 minutes. Sera which were to be tested were diluted 2-fold starting with a 1:10 dilution in glycine saline buffer to yield 0.5 ml in each tube. Clear test tubes measuring 10 x 75 mm were used. To each tube of diluted serum, 0.5 ml of the prepared antigen-latex-buffer mixture was added. Tubes were shaken, incubated for 1½ hours in 56°C water bath, centrifuged at 2300 rpm for 3 minutes and read. Appropriate nonspecific serum and antigen controls were included. Positive reactions were indicated by a clear supernatant, negative reactions by a turbid supernatant similar to that in the control tubes. *In bacterial agglutination test,* the same bacterial antigen was used as in the latex test. Serial dilutions of sera were made. To 0.2 ml of each dilution, 0.2 ml of antigen was added. The mixtures were incubated at 37°C for 2 hours, then left overnight at 4°C. Agglutination was read after gentle shaking.

Results. 1) *Investigation of conditions required for a satisfactory latex test.* *pH* of the system was found not to be too critical. The test worked well within pH range of 5 to 10. Below pH 4, spontaneous agglutination of the latex particles occurred. *Temperature of Incubation,* whether at 37°C or 56°C, seemed to have little or no effect upon the results when tests were read after 1½ hours. *Size of latex* (polystyrene) particles was critical. Reactions that were clear-cut when particles with a diameter of 0.81 μ were used, failed to appear when particles of 0.1 μ diameter size were substituted. (These particles were furnished by Dr. Jacques Singer.) *Amount of latex* used in each test had to be adjusted. If there was too little or too much, tests were difficult to read. Satisfactory *antigens* were saline-washed bacteria from thioglycollate cultures incubated at 37°C for 3-7 days. Several of these anaerobic diphtheroids agglutinated spontaneously when used as an antigen in the bacterial agglutination test, but when used in the latex test this difficulty did not influence the results. Some antigen could also be found in the supernatant of a saline suspension of these bacteria kept at 37°C overnight. Both these antigens were destroyed by boiling. However, if the bacterial suspension was first acidified, an extract could be prepared by boiling, as in the Lancefield method for streptococci(2). This extract contained an antigen which also reacted in the latex test with serum from a rabbit immunized with whole organisms. *Amount of antigen* had to be controlled. For example, in one experiment with the standard amount of bacterial antigen, the titer of a serum was 1/320; whereas when 3 times that amount of antigen was employed the titer fell to 1/80, and with ⅓ the amount of antigen, the reaction was unreadable. *Order of addition of reagents* was very important. Latex particles had to be first sensitized with the antigen—not with the immune serum.

TABLE I. Serological Reactions of Anaerobic Diphtheroids with Homologous and Heterologous Rabbit Antisera.

	Diphtheroids			
	M		G	
Serum	Aggl.	LA	Aggl.	LA
Normal	0	0	0	0
Anti-M	1/160*	1/160	0	0
Anti-G	0	1/160	0	1/640

Aggl. = Bacterial agglutination test.
LA = Latex " " .

* Highest dilution of serum giving positive reaction.

2) *Demonstration of immunological relationships by means of the latex test.* An example is shown in Table I. Rabbits were immunized by intravenous inoculation twice weekly of increasing amounts of suspensions of anaerobic diphtheroid strains, G or M, which had been recovered from patients at the North Shore Hospital. (Strain G had been isolated from the bone marrow of a child with an osteolytic bone lesion, and strain M had been recovered from the blood stream of an adult with arthritis and pericarditis.) When these strains were tested by means of the bacterial agglutination test with specific antisera only the M strain was agglutinated by the anti-M serum. G was not agglutinated by its homologous antiserum. However, when these organisms were examined by latex agglutination test, both G and M strains gave positive reactions with their homologous antisera. The anti-G serum also cross reacted with the M organism, although at a lower titer. This suggested the possibility of an immunological relationship between both anaerobic diphtheroids. Indeed, with the latex agglutination test it was possible to demonstrate an immunological relationship between many anaerobic diphtheroids. Table II summarizes the results of several experiments with the latex test in which anti-G serum was used with a variety of anaerobic diphtheroids and related bacteria. All of the typical anaerobic diphtheroids tested gave positive reactions. However, some strains after continued passage lost their ability so to react. As for the positive reaction with *C. acne,* others, using different technics(1) have also found that this organism may cross react serologically with anaero-

TABLE II. Results of Latex Agglutination Tests between Anti-Anaerobic Diphtheroid Strain G Rabbit Antiserum and a Variety of Microorganisms.

Organisms	Results	Organisms	Results
Anaerobic diphtheroids		Actinomyces	
G	+	*A. bovis*	0
M	+	*israeli*	0
E	+		
R_O	+	Others	
T	+	*Nocardia asteroides*	0
R_B	+	*Lactobacillus acidophilus*	0
MOR	+		
Corynebacteriae		*Propioni bacterium freudenreichii*	0
C. acne	+		
hoagii	0	*Staphylococcus aureus*	0
xerose	0		
diphtheriae	0	*Beta hemolytic streptococcus* (type 12)	0
pseudodiphtheriticum	0	*H. influenza* (type B)	0
pyogenes	0		

bic diphtheroids with which it probably shares a common antigen.

Discussion. Latex (polystyrene) particles which are now readily available have become useful serological tools. Singer and Plotz(3) first employed them in a test for rheumatoid arthritis. More recently, there have been reports of their value in study of histoplasmosis (4) and of trichinosis(5). These particles probably make more apparent a precipitinogen-precipitin reaction. Collodion particles were used for this purpose more than 30 years ago, but were of limited value since conditions for preparing and preserving them were very exacting and time consuming(6).

Singer and Plotz(3) stated that the "latex fixation" technic could also be used with Type VII antipneumococcal rabbit serum and Type VII pneumococcal polysaccharide. In this report, we have extended this observation to another bacterial antigen-antibody system, that of anaerobic diphtheroids and their specific antisera, and have investigated optimum conditions for this reaction. Other bacterial antigen-antibody systems, however, may require different conditions.

Summary. A technic for using latex (polystyrene) particles in a serological test with anaerobic diphtheroids is described. Optimum conditions under which latex particles may be used with this bacterial antigen-antibody system are reported. Evidence is presented to indicate a possible immunological relationship between various anaerobic diphtheroids.

The authors wish to acknowledge with thanks the capable assistance of Leona Caroline, Helen Lyons, and Dr. Liborio Garcia.

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Received May 2, 1960. P.S.E.B.M., 1960, v104.

Effect of Phosphorylated Hesperidin and Hyaluronidase on Rate of Erythrocyte Removal from Rat Peritoneal Cavity.* (25952)

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Phosphorylated hesperidin decreases net absorption rate of 0.9% sodium chloride solution from the peritoneal cavity of rats. Hyaluronidase abolishes this effect and by itself increases absorption rate in intact animals(1). The investigations reported below were conducted to determine whether phosphorylated hesperidin and hyaluronidase affect rate of absorption of erythrocytes from peritoneal cavity of rats.

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* Opinions expressed herein are those of the authors and are not to be construed as official or reflecting the views of the Navy Department or naval service at large.

† This work has been completed during senior author's tenure as Natl. Science Fn. Research Fellow at Wayne State Univ. Coll. of Med. and Detroit Receiving Hosp.

Materials and methods. Adult hooded female rats (175-200 g) raised at the Naval Med. Research Inst. were housed in individual cages in an air-conditioned room and had free access to laboratory rat chow and water. Experiments were performed as follows: 1) Animals were injected intraperitoneally (I.P.) with 15 mg of phosphorylated hesperidin (kindly donated by Dr. G. J. Martin, National Drug Co., Philadelphia) in 0.2 ml of 0.9% sodium chloride solution (isotonic saline) at 4 p. m. At 9 a. m. the next day they received a similar I.P. dose of phosphorylated hesperidin followed immediately by an I.P. injection of 6 ml of a suspension of erythrocytes. 2) Hyaluronidase (of bovine testes origin 1550 USP u/mg generously donated by Wyeth Inst. for Med. Research) was administered I.P. in 0.2 ml of isotonic saline, 6000 U.S.P. units per injection. The animals were injected at 9 a.m., 4 p.m. and the next day at 9 a.m. Immediately following last hyaluronidase injection the animals received I.P. 6 ml of erythrocyte suspension. Red blood cell suspension was administered through an 18 gauge needle under ether anesthesia, light enough so that the animals were awake within 2-3 minutes after completion of injection. No untoward reaction to I.P. injection of erythrocytes was observed. Groups of animals were sacrificed at various time intervals following injection of erythrocytes. The abdominal cavity was exposed and the remaining suspension recovered. The diaphragm, peritoneum, thoracic lymph vessels and lymph nodes were thoroughly inspected during autopsy. *Preparation of erythrocyte suspension.* On the day preceding an experiment, blood obtained by heart puncture from adult rats was collected in a syringe containing about 1 ml of 4% sodium citrate for each 10 ml of blood. The blood was centrifuged in a refrigerated centrifuge (4.5°C) at 2500 rpm for 30 minutes. The plasma and most of the buffy coat were discarded. The packed red cells were resuspended in an equal volume of normal saline solution and recentrifuged under refrigeration. The washing procedure was repeated 2 more times and after the last washing the cells were resuspended in an equal volume of isotonic saline and refrigerated until use. *Recovery of red blood cell suspension from peritoneal cavity.* Animals were sacrificed with an overdose of ether. Abdominal cavity was exposed, the remaining cell suspension removed and its volume measured. The abdominal cavity was rinsed twice with 5 ml of isotonic saline which was combined with the recovered suspension. Red blood cells were packed by centrifugation in a Kolmer graduated 10 ml centrifuge tube (Corning No. 8360) at 2500 rpm for 30 mintues. Volume of packed cells was read with an accuracy of 0.05 ml.

Results. Effect of phosphorylated hesperidin. In untreated animals volume of fluid and red blood cells recoverable from the peritoneal cavity decreased progressively with time. Rate of removal followed essentially a straight line for the first 4 hours, diminished between 4 and 8 hours after injection (Fig. 1). About 50% of the red blood cells were removed within the first 4 hours, and after 8 hours only 20% of originally administered volume could be recovered from the peritoneal cavity. In the hesperidin treated animals rate of removal of red blood cells and fluid was slow. Over 88% of injected erythrocytes could be recovered 8 hours after injection. Four hours after injection of the red blood cell suspension in untreated animals, diaphragm, thoracic lymphatics and lymph nodes were dark red (Fig. 2a). Microscopic examination of the

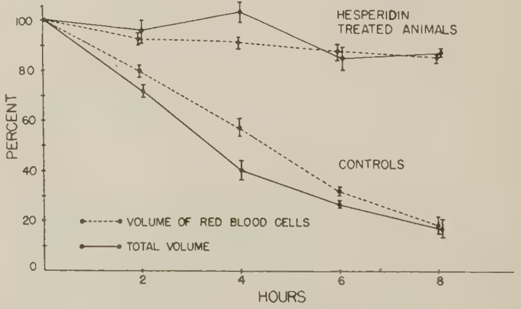

FIG. 1. Effect of two 15 mg injections of phosphorylated hesperidin on absorption of a 50% suspension of red blood cells in physiological saline from the peritoneal cavity of rats. Each point on the curves represents a mean from 12 animals.

TABLE I. Effect of Hyaluronidase on Absorption of Red Blood Cell Suspension (RBC) from Peritoneal Cavity of Rats.

	% of total vol and RBC vol recovered after inj. of 6 ml RBC suspension									
	2 hr		4 hr		6 hr		10 hr		17 hr	
Treatment	Vol*	RBC	Vol	RBC	Vol	RBC	Vol	RBC	Vol	RBC
Hyaluronidase and saline	56.8 ± 3.9†	82.2 ± 3.3	31.9 ± 1.4	52.4 ± 1.3	20.2 ± 1.8	33.2 ± 2.2	no free fluid	15.0 ± 1.6	no free fluid	9.0 ± 2.5
	(5)		(11)		(12)		(12)		(8)	
	P <.01	.1 > P >.5	P <.01	P <.01	P <.01	P <.05		P <.001		P<.01
Saline	73.2 ± 1.6	90.4 ± 2.6	40.4 ± 1.3	61.0 ± 2.1	28.0 ± 1.2	41.8 ± 3.0	*Idem*	31.0 ± 2.9	*Idem*	20.0 ± 2.2
	(5)		(12)		(12)		(10)		(8)	

* Fluid and RBC. † Mean ± stand. error. Numbers in parentheses indicate No. of animals.

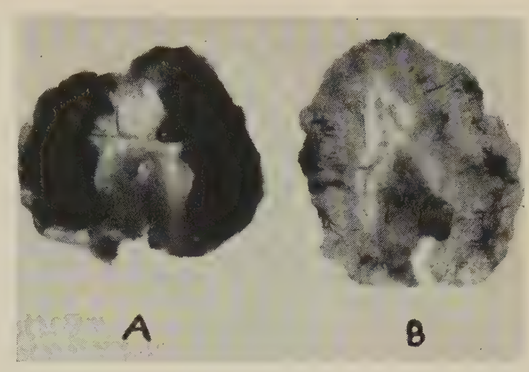

FIG. 2. Diaphragms of rats sacrificed 4 hr after intraper. inj. of red blood cell suspension. A. Normal control. B. Hesperidin treated.

material responsible for this discoloring revealed it to be red blood cells. In animals treated with phosphorylated hesperidin and sacrificed 4 hours after injection of a suspension of erythrocytes, the diaphragms were translucent, only a few small areas being filled with blood (Fig. 2b). Microscopic examination of the diaphragms (paraffin embedded and stained with Masson's Trichrom) did not reveal morphologic changes in the peritoneal structures.

Effects of hyaluronidase. Treatment with hyaluronidase produced a small but statistically significant ($P<0.01$) increase of absorption rate of the fluid portion of the red blood cell suspension at all time intervals after injection. The effect on absorption of erythrocytes was also slight. At each examined time interval the hyaluronidase treated animals had fewer erythrocytes recoverable from the peritoneal cavity than controls (Table I). Inspection of diaphragms revealed no difference between hyaluronidase treated and control animals.

Discussion. Absorption of blood from the peritoneal cavity of man and experimental animals was described in the 19th century. The early literature on this subject has been reviewed by Hunter(2). More recent studies revealed that the primary and probably only significant route of erythrocyte absorption is *via* the diaphragmatic lymphatics(2-5). From there the erythrocytes pass into the host's general circulation(6-8) where they survive for a normal life span(9,10). The mechanism of the absorption process, however, is poorly understood. Simer(11) concluded that small particles (India Ink) can pass from the peritoneal cavity of rats into the subperitoneal lymphatics of the diaphragm between the contiguous borders of the mesothelial cells of the peritoneum and endothelial cells of the lymph vessels. According to Allen(12) the erythrocytes pass through a triple layered membrane (peritoneal mesothelium, a fenestrated basement membrane and endothelium of the lymphatics) separating peritoneal cavity from diaphragmatic lymphatic spaces. The mesothelium and endothelium stretch during expiratory movements of the diaphragm, and openings ("stomata") are formed (up to 25.5 μ in diameter) through which the suspension of particular matter is "sucked" into the lymphatic channels.

Courtice *et al.*(5) demonstrated that in rats erythrocytes are removed from the peritoneal cavity at a relatively rapid rate, up to 90% of injected erythrocytes being removed within 10 hours. In our experiments rate of removal of erythrocytes was similar in intact animals. However, in phosphorylated hesperidin treated rats, erythrocytes were retained in the peritoneal cavity for periods of up to 8 hours after injection. There was also a block in absorption of the solution in which red blood cells were suspended, confirming previous report by Steinberger *et al.*(1).

Diaphragms of untreated controls were packed with red blood cells while diaphragms of hesperidin treated animals were essentially devoid of grossly demonstrable red blood cells; this suggests that the block was effected at the membrane dividing peritoneal cavity from lymphatic channels of the diaphragm. Considering the mechanisms of erythrocyte removal from the peritoneal cavity proposed in the literature(11,12), hesperidin would appear to exert its effect upon the peritoneal membrane either by inhibiting its ability to form openings during the respiratory excursion of the diaphragm, or by modifying the ground substance between the contiguous borders of the cells, making it less permeable. The effect on the peritoneal structures appears to be local since pilot experiments employing subcutaneous administration of hesperidin did not reveal any effect on peritoneal absorption. Histological examination of the peritoneum revealed no structural changes. A study of a direct local effect of hesperidin on connective tissue is in progress.

The effect of hyaluronidase on passage of red blood cells from the peritoneal cavity was small, but statistically significant. This may indicate that permeability of the membrane was at its peak before treatment with hyaluronidase, and further increase was difficult to achieve. Another, more plausible explanation would be that the lymphatics through which the blood cells are removed have a maximum volume capacity which is the rate-limiting factor not influenced by an increase in membrane permeability. This interpretation is supported by the fact that in untreated control animals the entire lymphatic system engaged in removal of the red blood cells is actually packed with them. The lymph from these lymphatic channels has been reported to contain up to 16 million red blood cells per cmm and to form a thick sludge(12).

Summary. Rate of absorption of erythrocytes from peritoneal cavity of rats was found to be similar to that reported in the literature. Absorption was almost completely blocked by phosphorylated hesperidin. Evidence was presented suggesting that the block is achieved at the membranes separating peritonal cavity from lymphatics of the diaphragm. Hyaluronidase increased rate of absorption only slightly; however, this increase was statistically significant.

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Received May 10, 1960. P.S.E.B.M., 1960, v104.

Genetic Susceptibility to Goldthioglucose-Induced Obesity in Mice.[*] (25953)

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A single injection of goldthioglucose at appropriate concentrations produces a persistent obesity in mice(1,2,3,4,5). The weight gain is correlated with an increase in food intake (6). Lesions found in the hypothalamus of goldthioglucose-injected mice are considered to be the basis for the hyperphagia and ensuing weight gain(7,8,9), and localization of tissue destruction in ventromedial nuclei of the hypothalamus has been related to the postulated existence of a "glucoreceptor" center in this particular area of the diencephalon(7). However, lesions have been found in areas of the brain that lie outside the confines of the hypothalamus following an obesity-inducing dose of goldthioglucose(8,10). Liebelt and Perry(8) have shown a genetic difference in the susceptibility of CBA and C$_{57}$ Black mice to goldthioglucose-induced obesity. The purpose of this investigation was to extend these findings to other strains of inbred mice, thus studying the influence of genetic factors in goldthioglucose-induced obesity.

Methods. All strains of mice used in these experiments had been inbred by brother-sister mating for over 20 generations,[†] and included C$_{58}$, CBA, DBA/2, BALB/C, RIII as well as the following F$_1$ hybrids: (RIII x CBA), (CBA x RIII), (CBA x C$_{58}$), (C$_{58}$ x CBA), (CBA x BALB/C). Only male mice were used and were housed in groups of 4-6 animals per wooden cage (6 x 12 x 6 in.). Purina lab chow was fed *ad libitum.* Crystalline goldthioglucose (Solganol B)[‡] was dissolved in saline, and injected intraperitoneally at 90-110 days of age. All injections were made between 9:00 and 11:00 a. m.; the animals were not starved prior to treatment. A minimum of 10 and a maximum of 40 animals were injected at each of the various dose levels tested for each strain and F$_1$ hybrid combination; mean weight gain of the surviving animals was determined at 60 days post-injection. Susceptibility to the *toxic effects* of goldthioglucose was expressed only as the LD/100 dose.

Results. Table I shows that both gain in body weight and lethal toxic effects in any given strain vary with dose of goldthioglucose injected. C$_{58}$ mice appear to be most "susceptible" to goldthioglucose in terms of weight gain and toxicity as compared to the more "resistant" BALB/C, RIII and DBA/2 strains. Mice of the CBA strain fell within these two extremes. Weight gain response and mortality were characteristically variable, especially as LD/100 dose was approached, in all except the CBA strain. CBA mice appeared to be unique in that a uniform weight gain response could be predicted for each dose level over a range of 0.2 to 0.6 mg/g body weight with less than 10% mortality at the latter dose.

In general, all animals showed a greater weight gain as dose of goldthioglucose was increased. However, as the LD/100 dose was approached in the DBA/2, RIII, and BALB/C strains, animals actually lost weight when

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[*] Supported by grants from Am. Heart Assn. and U.S.P.H.S.

[†] Mice obtained from Kirschbaum Memorial Research Lab., Dept. of Anatomy, Baylor Univ. College of Medicine, Houston, Texas.

[‡] Courtesy of Schering Corp., Bloomfield, N. J.

TABLE I. Body Weight Gain (g) and Mortality following Various Concentrations of Goldthioglucose (mg/g Body Weight) at 60 Days Post-Injection.

Strain	Control	.1	.2	.3	.4	.6	.8	1.0	1.2	1.4	1.5
C_{58}	3.0 ± .52* (20)	7.33 ± 1.72 (18)	11.33 ± 1.66 (15)	10.16 ± 2.43 (21)	All dead (25)						
CBA	2.5 ± .50 (30)	2.0 ± .82 (30)	9.62 ± 1.21 (40)	13.25 ± 2.23 (40)	22.50 ± 1.52 (40)	23.12 ± 1.32 (20)	All dead (15)				
RIII	2.75 ± .83 (15)				3.0 ± .12 (16)	6.33 ± 1.24 (17)	15.0 ± 4.0 (20)	2.0 ± 1.2 (14)	All dead (10)		
DBA/2	2.83 ± .62 (15)					5.75 ± 1.32 (15)	14.0 ± 2.37 (18)	15.5 ± 5.31 (20)	4.2 ± 4.81 (12)	All dead (10)	
BALB/C	3.62 ± .42 (20)		3.75 ± .96 (18)		4.25 ± 1.68 (12)	7.62 ± 2.82 (14)	10.25 ± 3.12 (14)	12.50 ± 2.68 (12)	13.0 ± 2.89 (13)	3.2 ± .41 (10)	All dead (12)

* Stand. dev. No. of mice in parentheses.

compared with controls. Some of these animals have remained alive as long as 8 months post-injection at these reduced weights. Animals dying within 2-3 days post-injection besides demonstrating extensive hypothalamic damage and gastric ulcers also showed tubular necrosis in the kidney and parenchymal destruction in the liver.

All F_1 hybrid mice responded to a dose range of goldthioglucose similar to that characterizing CBA mice (Table II). This was found whether the CBA genetic influence was of maternal or paternal origin. Maximum mean weight gain at 60 days post-injection was 28.4 g for (RIII x CBA) F_1 hybrids, which was greater than maximum body weight attained by either of the parent stocks.

Discussion. The facility with which obesity may be induced in genetically different strains of mice is of both biological and practical importance. C_{58}, DBA/2, RIII and BALB/C mice showed a relatively unpredictable and variable weight gain and mortality as compared to the CBA strain over the dose range of administered goldthioglucose. Brecher and Waxler(1) indicated that of albino mice injected with an LD/50 dose (0.8 mg/g body weight) of goldthioglucose only ⅓ of the surviving animals became obese. Mortreuil-Langlois(11) reported that 1.5 mg/g body weight was necessary to induce a significant, yet variable weight gain in the XVII strain of mice, and LD/100 dose ranged between 1.6 and 1.7 mg/g body weight. Inbred strains of mice are considered to be genetically identical within a given subline; variation in response to goldthioglucose in these particular strains suggests the operation of non-genetic factors in addition to genetic determinants. The nature of such factors necessitates further study. The uniform response of CBA mice to the effects of goldthioglucose appears to be unique. It would be of interest to determine whether sublines of this strain in other laboratories respond similarly.

The susceptibility to the toxic effects of goldthioglucose expressed as LD/100 dose also varied with the different strains. Animals dying within 2-3 days post-injection demonstrated gastric ulcers and extensive hypothala-

TABLE II. Body Weight Gain (g) and Mortality in F_1 Hybrids following Various Concentrations of Goldthioglucose (mg/g Body Weight) at 60 Days Post-Injection.

Strain	Control	.1	.2	.4	.6	.8
RIII ♀ × CBA ♂	4.2 ± .6* (10)	4.1 ± .9 (15)	7.23 ± 1.05 (13)	17.3 ± 2.19 (12)	28.4 ± 2.02 (10)	All dead (10)
CBA ♀ × RIII ♂	3.9 ± .5 (10)			19.4 ± 1.86 (16)	25.6 ± 2.26 (13)	All dead (14)
CBA ♀ × C_{58} ♂	4.0 ± .72 (10)	5.1 ± .9 (18)		18.3 ± 2.25 (12)	All dead (11)	
C_{58} ♀ × CBA ♂	4.1 ± .64 (10)	4.8 ± .44 (17)		15.4 ± 1.87 (14)	All dead (14)	
CBA ♀ × BALB/C ♂	3.3 ± .9 (10)			12.3 ± 2.1 (12)	23.4 ± 2.2 (14)	All dead (12)

* Stand. dev.

No. of mice in parentheses.

mic damage(8), as well as severe tubular necrosis of the kidney and parenchymal destruction in the liver. The latter type of lesions could account for the marked weight reduction seen in certain animals of DBA, RIII and BALB/C strains at dose levels approaching LD/100.

All F$_1$ hybrid combinations showed a weight gain response similar to that of the CBA strain for a given dose irrespective of whether the other parental strain was "resistant" or "susceptible." The LD/100 dose for F$_1$ hybrids was also similar to that of the CBA parent. It would appear that genetic factors involved in goldthioglucose obesity, and derived from the CBA parent, are expressed as the dominant influence in F$_1$ hybrids.

The 5 inbred strains of mice showed some variation as to maximum body weight attained for the range of doses of goldthioglucose utilized. (RIII x CBA) F$_1$ hybrids showed a weight gain to a given dose of goldthioglucose greater than that seen in either of the parent strains. These data suggest the operation of genetic and/or non-genetic factors which may influence extent of hypothalamic involvement by a given amount of goldthioglucose or may regulate peripheral factors involved in lipid deposition.

Thus the site(s) of gene action involved in "susceptibility" or "resistance" to goldthioglucose-induced obesity must be investigated with respect to 1) metabolism of goldthioglucose in organs other than the brain, 2) development of lesions in the hypothalamus and other areas of the brain, and 3) peripheral response of various organs including the fat depots to the induced hyperphagia.

Summary. Five inbred strains of mice (C_{58}, CBA, RIII, DBA and BALB/C) and 5 F$_1$ hybrid combinations were studied with respect to effects of single injections of various concentrations of goldthioglucose on body weight gain and lethal toxicity. Gain in body weight and mortality following a single injection of goldthioglucose was dependent on strain of mice and amount injected. In general, there was a greater increase in weight gain when larger doses of goldthioglucose were administered. The C_{58}, RIII, DBA and BALB/C strains of mice showed a relatively variable weight gain and mortality as compared to the CBA strain over the dose range of administered goldthioglucose. Mice of the latter strain were unique in that they showed a uniform response with respect to these 2 parameters. The F$_1$ hybrid mice showed a weight gain response and a LD/100 dose comparable to that of the CBA strain.

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Received June 2, 1960. P.S.E.B.M., 1960, v104.

A Pneumotropic Line of Lymphocytic Leukemia from Inbred Princeton Mice. (25954)

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Lymphocytic leukemia (lymphosarcoma) occurs naturally in adult Princeton mice (Pr, PRI, or P) from the random-bred colony maintained at the Rockefeller Institute. The disease is sporadic with a morbidity rate of 3.1% in 1050 discarded breeders, 10 to 12 months old, killed and autopsied during the past 7 years. Six of the 33 cases of leukemia (2.7%) were recorded in 215 males and 27 (3.2%) in 835 females. A branch of the Princeton strain with a high rate of leukemia, 80 to 90% in old adults, was established by Dr. Clara Lynch in 1946(1). This branch, designated PL or PrL, was inbred for more than 20 generations. In connection with other studies on murine leukemia it was of interest to determine the behavior of the PrL line in random-bred Princeton mice. Differences in activity of neoplastic cells from the 2 lines, one with low morbidity under natural conditions and the other high, were brought out in transmission experiments and are presented here. The naturally acquired leukemia of adult mice from the random-bred Princeton colony is characterized chiefly by marked enlargement of regional lymph nodes and spleens. The disease is reproducible in weanlings from the same colony by intraperitoneal injection of organ suspensions from naturally affected adults and is transmissible by serial passage. On repeated passage in recently weaned mice hepatitis virus may appear and alter the leukemic reaction.

Materials and methods. Random-bred female weanlings under 15 g in weight, usually 10 to 12 g, were used in most of the following experiments. The PrL line was discontinued by Dr. Lynch in 1951 but a subline was maintained by Dr. Wilhelmina F. Dunning at Univ. of Miami, Fla. We are indebted to her for a supply of young PrL mice with transplanted leukemia. Suspensions for intraperitoneal injection were prepared from pooled mesenteric lymph nodes and spleens of 2 or more leukemic mice. A bulky but unmeasured tissue mince, finely cut with scissors. was added to 2 or 3 ml of saline. After standing for a brief period the turbid supernatant was removed and constituted the inoculum. Similar suspensions were prepared from lungs and thymus for nasal instillation. Lymphocytes were verified by phase microscopy. In the presence of hepatitis virus liver minces were homogenized in a glass tissue grinder with sufficient saline to make a concentration of about 10%. Testing of suspensions was carried out in groups of 5 or 6 mice. Intraperitoneal injections were made with an inoculum of .1 to .15 ml and nasal instillation, following ether anesthesia, with about .05 ml. The injected mice were usually held under observation until one or more deaths had occurred. At this time all survivors that showed signs of illness were killed with ether and autopsied.

Results. A passage series begun in weanlings with an organ suspension from a random-bred leukemic adult was maintained for 40 transfers in 240 mice. The interval between injection and onset of external signs varied from 25 days in the first passage to 7 days after continued transfer. Mortality rate was 25% and morbidity rate 88%. At autopsy regional lymph nodes, particularly the mesenteric, were enlarged and generally colorless. Spleens were hyperplastic and often pallid. Involvement of liver, kidneys and ovaries also occurred but was more variable. Free fluid was occasionally present in the peritoneal cavity. Lungs were consistently normal and thymus pale. The invasive cell in sections of leukemic organs was a lymphocyte. All passage series made with similar suspensions during the past 7 years have conformed to this general pattern.

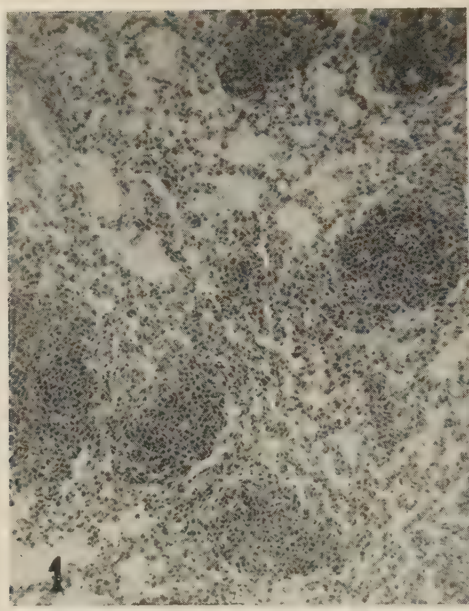
None of the mice in this series showed any evidence of murine hepatitis. The number of passages which may elapse before appearance of the causal virus is unpredictable and highly variable. In a later series necrotic lesions were detected in the liver after 8 passages and a virus of low virulence recovered. Unlike the virus reported in 1952 (MHV Pr) this strain was rarely lethal on intraperitoneal injection in weanlings(2). In presence of the virus the morbidity rate from leukemia was not appreciably affected but the pathologic reaction was less marked and somewhat delayed.

A similar passage series was begun in random-bred weanlings with a spleen and lymph node suspension from 2 leukemic mice of the inbred PrL branch. Thirty-six successive transfers were made in 216 females at intervals of 6 to 8 days. Morbidity and mortality rates were 94 and 31%, respectively. The macroscopic pathologic response was noticeably different from that of the preceding series. Hyperplasia of regional lymph nodes was accompanied by congestion resulting in a pink or light red coloration. In some mice the axillary and inguinal nodes were surrounded by a wide red zone of subcutaneous injection. Spleens were commonly enlarged and somewhat darker in color than normal. Involvement of livers, kidneys and ovaries also occurred but was not constant. Lungs were usually affected and showed pink areas of stippling or streaking. This reaction was best observed in the gross with a low power dissecting microscope.

Proliferation of the neoplastic cells in the lungs was clearly indicated on histological examination. Infiltration of the alveoli by the leukemic lymphocytes resulted in formation of discrete compact islands (Fig. 1). Perivascular and peribronchiolar cuffing also occurred. Interstitial tissue was somewhat distended by erythrocytes and lymphocytes. Erythrocytes and, less regularly, fluid deposits were present in some of the alveoli. In more advanced cases, characterized by areas of frank consolidation, the normal architecture of the lung was obliterated by extensive sheets of closely packed tumor cells.

In the 36th passage necrotic foci appeared in the liver of one mouse and hepatitis virus of low virulence was subsequently recovered. Ten additional spleen passages were made. Results were variable but indicative of interference in development of the leukemic cells. Mortality rate of 60 mice was 42%. Thirty of the 34 survivors, killed on the 7th day after injection, showed liver lesions (necrotic foci, hemorrhages and pits) pathognomonic of murine hepatitis. Twenty-one of the survivors also showed leukemic manifestations which were somewhat less advanced than those observed prior to onset of hepatitis. A liver section in which an extensive area of necrosis is adjacent to a cluster of the leukemic cells is presented in Fig. 2.

Macroscopic appearance of lungs from the 2 leukemic PrL mice was suggestive of the pulmonary reaction produced in mice by viruses of grey lung pneumonia and wild rat pneumonia(3,4). The resemblance was subsequently shown to be purely superficial by histological examination but it prompted injection of lung suspensions by the nasal route. Cell-free supernatants were innocuous on nasal instillation but whole suspensions resulted in pulmonary as well as generalized leukemic lesions. Forty passages were made in 218 random-bred weanlings. The respective morbidity and mortality rates were 82 and 38%. The interval between injection and onset of leukemic symptoms was longer and more variable than that following intraperitoneal injection. Average interval was 16 days, with a minimum of 11 and a maximum of 29 days. Ninety-two mice were killed and autopsied



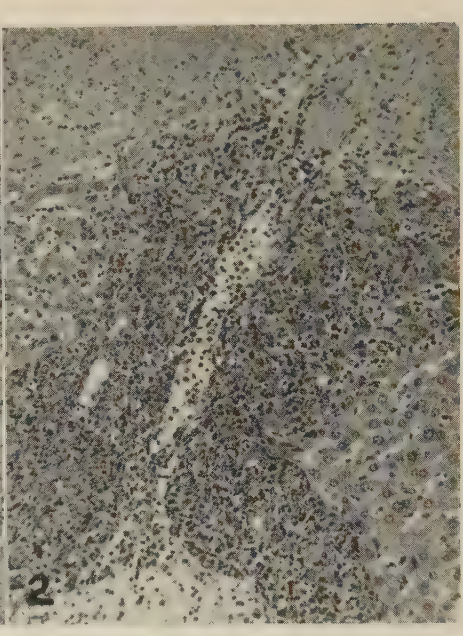


FIG. 1. Photomicrograph of lung removed on 7th day from a Princeton mouse inj. intraper. with leukemic cells of PrL line. Compact groupings of neoplastic cells and interstitial thickening. HE 125×.

FIG. 2. Photomicrograph of liver removed on 7th day from Princeton mouse inj. intraper. with spleen suspension of PrL line. The margin (upper) of a large necrotic focus is bordered by infiltrating leukemic cells and a small area of normal liver parenchyma. HE 125×.

shortly after outward signs of illness were observed. Pulmonary involvement was constant and usually accompanied by hyperplasia of spleens and lymph nodes. It may be noted that Princeton mice from our colony are free from intercurrent respiratory disease. The red coloration of all leukemic organs except the kidneys was particularly striking in the mice of this series.

None of the mice injected intranasally showed any indication of hepatitis at autopsy. Following appearance of the causal virus in the mice of the preceding intraperitoneal series a second one was begun with a lung suspension from the 20th nasal passage. Sixty-eight successive spleen and lymph node transfers have now been made in weanlings in absence of hepatitis. Free fluid containing the leukemic cells was first observed in one mouse of the 36th passage and by the 50th one was a nearly constant finding. In the presence of fluid, leukemic response of the abdominal organs was noticeably retarded.

Attempts to induce pulmonary lesions with the low leukemic line of random-bred Princeton mice were uniformly unsuccessful. In these experiments suspensions of lungs or spleens and lymph nodes from mice of the intraperitoneal series were injected intranasally. Lungs of the injected mice, some of which were held for 2 months, were regularly normal. Nasal instillation of one spleen and lymph node suspension was attended, however, by leukemic involvement of regional and abdominal lymphoid organs. The neoplastic cells were maintained for 2 additional nasal passages but were not demonstrable thereafter.

Discussion. In reviewing the earlier work on lymphocytic leukemia in mice Dunn stressed the individual and recurring pattern of organ involvement and noted that the lungs were sometimes extensively invaded(5). The observations of Furth, Seibold, and Rathbone and of Furth on pulmonary infiltration in leukemia are noteworthy(6,7). Furth appears to have been the first to induce tumor growth in lungs of mice by nasal instillation of neoplastic cells(7).

Our autopsy records of the low level leukemia in random-bred Princeton mice number 33 natural cases and some hundreds of experimental ones. In no instance was a pulmonary reaction observed macroscopically at autopsy. The pathogenicity and invasiveness of the neoplastic cells were evidently altered during their residence in inbred mice. How or when these changes occurred is a matter of speculation. On transmission to random-bred mice the altered cellular behavior was indicated by an increased toxic reaction in lymphoid tissue and by regular proliferation in the lungs. The latter change was so constant on both intraperitoneal and intranasal injection that the term pneumotropic line seemed appropriate.

In view of the present emphasis on viruses in the etiology of certain murine tumors including the leukemias the possible role of the respiratory tract in outward dissemination of these agents may be worthy of consideration.

There was no apparent difference between the 2 leukemic lines in regard to their effect on emergence of murine hepatitis virus in Princeton weanlings following intraperitoneal injection. Similar viruses were obtained with both lines. There was reason to believe, however, that nasal instillation of the high level leukemic cells provided a means of avoiding the liver lesions produced by these latent agents.

Summary. 1) Lymphocytic leukemia, of sporadic occurrence in random-bred Princeton mice (3.1% in 1050 adults), was transmitted to weanlings by intraperitoneal injection of lymphoid organ suspensions and maintained by serial passage. Spleens and regional lymph nodes were commonly enlarged but lungs were not involved. 2) A high level leukemia line from inbred Princeton mice, originally established by Dr. Clara Lynch, was similarly maintained in random-bred weanlings. Neoplastic cells also proliferated in lymphoid tissue, though with evidence of increased toxicity, and also invaded the lungs. Pulmonary lesions as well as generalized ones were regularly produced by intranasal injection of cells from high leukemia line but not from the low level one. 3) Necrotic lesions of liver appeared in one passage series with each of the 2 lines and murine hepatitis viruses of low virulence were recovered.

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Received June 6, 1960. P.S.E.B.M., 1960, v104.

Overwintering of Western Equine Encephalitis Virus.* (25955)

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The mechanism of maintenance of Western equine encephalitis virus in nature during non-epidemic periods and particularly during cold winter months has puzzled many investigators. This virus has been isolated from many species of birds(1,2,3) and a chronic viremia has been produced in experimentally infected wild birds from 1 to 10 months duration(4). This finding has suggested the possibility of an overwintering reservoir for the virus. In studies of 1503 birds, Kissling *et al.* (5) failed to isolate virus from wild birds either in winter or spring, but during the summer when mosquitoes were active virus infected birds were present. Although WEE virus has been isolated repeatedly from pools of mosquitoes collected during practically every month of the year in temperate cli-

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* Supported in part by grant from Dept. of HEW, PHS.

mates(6), there appears to be only one successful isolation from mosquitoes collected during winter months in an area of colder climate. Blackmore and Winn(7) found WEE virus in one pool of 50 female *C. tarsalis* mosquitoes taken from mine shafts in Colorado. The pool showing virus was one of 62 prepared from mosquitoes collected during Dec., Jan., and Feb. A total of 1361 females were obtained under conditions of cold weather in Colorado. Some authors(8,9) have discounted the importance of the surviving female *C. tarsalis* as a means of overwintering of WEE virus. Rosenbusch(10) was able to infect several species of snakes with WEE virus. He infected a largato snake (*Tupinambis teguixin*), a *Bothrops amnodytoides* snake and one *Bothrops alternata* maintained the virus in the brain for 37 days but not in the blood. Two *Phylodryas culebra* snakes injected with WEE virus failed to harbor virus in the brain or blood. Thomas *et al.*(11) were able to infect common garter snakes (*Thamnophis* spp.) by either direct injection of virus or by allowing infected *Culex tarsalis* mosquitoes to obtain a blood meal. Virus was maintained by one of these snakes for 36 days. Lennette *et al.*(12) has suggested that squirrels may play a role in maintenance of the virus, the virus having been found in the nervous tissue of wild squirrels caught during the summer. During 1958 the majority of the state of Utah experienced an unusually hot, dry spring, and early summer necessitating more irrigation than usual. *C. tarsalis* mosquitoes began to appear in increased numbers in May and into the summer months(13). Cases of Western equine encephalitis were first reported in horses in June and human infections were evident beginning in July. A total of 58 proven human cases with 6 deaths were reported. This paper reports results of a continuing study on the mechanism of overwintering of WEE virus in nature.

Materials and methods. Snakes (*Thamnophis* spp.) were collected, beginning in April and each month through July, 1959. In preliminary hibernating experiments 4 garter snakes (*Thamnophis* spp.) were injected intraperitoneally with 0.25 ml of Western equine encephalitis virus (LD_{50} $10^{-5.5}$ in suckling mice) diluted 10^{-1}. The snakes were kept at 4°C for 17 days (July, 1959) when they were then bled and sacrificed for nervous tissue. Twenty-one *Thamnophis* spp. snakes were injected intraperitoneally with 0.25 ml of WEE virus (LD_{50} $10^{-6.5}$ in suckling mice) Dec. 11, 1959, then placed in outside hibernating cages simulating natural hibernation conditions. The cages were buried 3 feet deep underground with burrows to the surface. Outside temperatures varied from +47°F to –1°F during the winter. Temperatures inside the underground cages varied from 15 to 50°F. Relative humidity varied from 31 to 83%. Five uninoculated controls were placed in the same outside cage which housed the inoculated animals. Bleedings were done Dec. 30, 1959, Feb. 13, Mar. 12, and Apr. 28, 1960. One-tenth ml of blood was added to 0.9 ml sterile skim milk and inoculated into suckling mice within 5 minutes after blood samples were obtained. The milk-blood mixtures were then held at –28°C and bloods showing positive virus content were then titrated in suckling mice.

Neutralization tests were carried out using known WEE positive immune serum and virus from snake bloods. The immune serum was held constant (1:10) and the virus was diluted with sterile skim milk. After 2 hour incubation at 37°C the mixtures were then injected intracerebrally into suckling mice in doses of 0.025 ml.

Results. Virus (WEE) was isolated from the blood of hibernating snakes 19, 64, 92, and 139 days after inoculation and then hibernating during the winter months. Virus decreased in quantity as the winter came to a close, but an increase of virus was present beginning with warm weather in April in not only a greater number of the snakes blood, but the titer increased. Results are presented in Table I.

Six inoculated snakes died during the hibernation period, 5 of which strangled in the underground wire cages. One developed a necrotic tail and was not bled in March nor April. Three of the uninoculated controls died during the hibernation period. No evidence of illness was noted in any snakes. No

TABLE I. Results of Overwintering of WEE Virus in Hibernating *Thamnophis* spp. Snakes. Injected Dec. 11, 1959 with 0.2 ml intraper., mouse brain WEE virus (LD_{50} $10^{-6.5}$).

Bleeding date and days after inj. of WEE virus		Snake No.	Virus isolated from blood of snakes, and titer	
12/30/59	19 days	1, 2, 3, 4, 5 Control 26	1, 5	(10^{-1})
			3	(10^{-5})
			4	(10^{-3})
2/13/60	64 days	2, 3, 4, 5, 7, 8, 9, 10	2, 3, 4, 5, 7	(10^{-1})
			10	(10^{-3})
3/12	92 days	3, 4, 5, 8, 9, 11, 13, 14, 15, 17, 18, 19, 20, 21 Controls 26, 27	13	(10^{-1})
4/28	139 days	2, 3, 4, 5, 7, 8, 9, 11, 15, 16, 17, 18, 19, 21	15, 21	(10^{-1})
			4	($10^{-1.5}$)
			11	(10^{-2})
			5	($10^{-2.2}$)
			2	(10^{-3})
			19	($10^{-3.2}$)
			3, 7	($10^{-3.5}$)

virus was isolated from the blood of the controls tested.

Of the 4 snakes inoculated with WEE virus and hibernated in the refrigerator (4°C) virus was isolated from the blood but not from the brain of 2; in one, virus was demonstrated in the brain tissue, but not in the blood. No virus was demonstrated in either the blood or nervous tissue of one snake.

Virus from the snake blood was proved to be WEE by neutralization tests, using specific immune serum. The neutralization index of this immune serum was over 10^3.

Discussion. In preliminary experiments common garter snakes (*Thamnophis* spp.) have been found to maintain WEE virus for at least 139 days during winter hibernation. The outside hibernation cages simulated the normal overwintering habitat of these snakes. The data have indicated that the titer of virus in the blood of these inoculated snakes decreases during the cold months of the year. With warmer temperatures in the spring, however, the viral content of the blood of these snakes increased. This carry over of virus suggests that snakes may become a source of an infective blood meal for spring and summer hatched mosquitoes.

For epidemic or endemic cases of Western equine encephalitis to develop, there must be a source of virus for mosquitoes to initiate such infections. For mosquitoes to obtain an infective blood meal, there must be a source of blood from an animal which exhibits a viremia at the time of the mosquito feeding. Such an animal must carry the virus during the winter months and exhibit a viremia in the spring of sufficient proportion to enable the vector mosquitoes to obtain an infective blood meal.

From results presented, the common garter snake appears to fulfill the above suggested requirements of overwintering WEE virus in nature and are capable of development of a viremia in the spring. From initiation of infection of the first few mosquitoes in the spring, secondary virus hosts such as birds, could expand the virus source for more mosquitoes to become infected, thus producing a high infection in the mosquito and bird population. With a high population of infected vector mosquitoes during the summer months, horses and man become an accidental host for the virus.

Summary. 1) Adult garter snakes (*Thamnophis* spp.) inoculated intraperitoneally, overwintered WEE virus for at least 139 days. There was a steady decline in virus, with only 1 of 14 showing a viremia after 92 days hibernation. With warm spring weather, (139 days after virus inoculation) 9 of 14 animals exhibited a viremia with blood titers varying from 10^{-1} to $10^{-3.5}$. 2) Two of 4 garter snakes inoculated with WEE virus and kept at 4°C, for 17 days harbored the virus in the blood, but not the brain. One snake harbored the virus in the brain but not in the blood. No virus was detected in either blood

or brain after 17 days at 4°C in one of the snakes.

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Received June 8, 1960. P.S.E.B.M., 1960, v104.

Erythropoietic Stimulating Action of Acetylcholinesterase. (25956)

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This author reported production of experimental hyperchromic anemia in dogs by daily injection of acetylcholine(1). Liver extract or folic acid injections caused remission of this anemia with a concomitant rise in serum cholinesterase activity which was postulated to be the cause of remission. Other experiments(2) indicated that cobalt injection or production of marked anemia by phenylhydrazine caused significant increases in serum cholinesterase activity. Both of these procedures increase plasma erythropoietic stimulating factor (ESF or erythropoietin)(3,4,5). These facts suggested the possibility that cholinesterase, or part of its molecule, might be concerned with erythropoiesis, and prompted the present investigation.

Procedures and methods. Injections of Acetylcholinesterase* (200 units daily) were given subcutaneously to each of 7 normal rats weighing about 180 g. Another group of 7 untreated rats served as controls. Blood samples were obtained by cutting off the tip of the tail, before and at various intervals after commencement of daily injections. Erythrocyte counts and reticulocyte percentages were determined by standard methods, and hematocrit percentage of packed cells was determined using capillary tubes and a Clay-Adams micro-hematocrit centrifuge.

Results. Table I shows results of daily subcutaneous injections of 200 units (*i.e.,* 4 mg of commercial preparation in 1 cc of water) of Acetylcholinesterase into 7 normal rats. After 5 to 7 daily injections, reticulocyte percentages were elevated to 6.1% (average), which represented a 5-fold increase over average pre-experimental value. Erythrocyte counts were not significantly increased at this time, but after 2 weeks of daily injections, they had increased by 18% (average) or 1.4 million cells per cmm of blood. Hematocrit percentages of packed cells did not increase significantly at any time. The control group of 7 untreated rats showed no significant changes in the blood throughout period of experiments. Observations performed on only 2 experimental animals indicated that Acetylcholinesterase injections increased hemoglobin values about 10%, but caused no significant changes in total leukocyte counts.

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* Acetylcholinesterase from bovine erythrocytes contained 20,000 Ammon units in 400 mg of material. (Nutritional Biochemicals Corp.)

TABLE I. Effect of Acetylcholinesterase on Blood of Rats. 200 units given daily to each experimental rat.

Group	Time of blood sampling	Reticulocytes, %	Erythrocytes in millions/mm³	Hematocrit, % cells
Exp. (7 rats)	Before start of inj.	1.2 (.8-1.7)*	7.90 (7.40-8.50)*	45.7
Control (")	Before start of exp.	1.1 (.6-2.2)	8.25 (7.50-8.75)	46.7
Exp.	After 5-7 daily inj.	6.1 (3.4-9.5)	8.15 (7.65-8.77)	45.9
Control	Untreated	1.2 (.8-1.8)	8.18 (7.51-8.55)	46.2
Exp.	After 12-16 daily inj.	2.3 (1.2-4.6)	9.30 (9.11-9.44)	46.3
Control	Untreated	1.3 (.8-2.0)	8.07 (7.76-8.78)	45.9

* Range given in parentheses.

Discussion. Acetylcholinesterase administration caused a marked increase in rate of formation or release of reticulocytes and erythrocytes. The fact that hematocrit values were not significantly changed, in spite of an increase of erythrocyte numbers, indicates that the Acetylcholinesterase causes an increased production of cells that are smaller than average in volume. Our present data do not indicate whether or not cholinesterase activity bears any relationship to plasma erythropoietic stimulating factor, although such a possibility is not precluded. Experiments are being continued in the effort to clarify this question.

Summary. Daily subcutaneous injections of 200 units Acetylcholinesterase into rats caused stimulation of erythropoiesis demonstrated by significant reticulocytosis within 7 days and increased erythrocyte counts after 14 days. Hematocrit values did not change significantly.

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Received June 14, 1960. P.S.E.B.M., 1960, v104.

Effects of Acute Hypoglycemia on Rat Testis. (25957)

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Hypoglycemia is known to exert a deleterious effect on certain endocrine glands and on cerebral structures(2,3,4) but no effect has been reported on male gonads. As these are known to consume glucose mainly(1), we attempted this histological study of the testis of rats submitted to acute hypoglycemia.

Material and methods. 318 male albino rats were used. They were separated as follows: A) Animals in which one hypoglycemic coma was induced: *Group 1*: 62 prepubertal rats weighing 35-50 g; 28 received 200 or 400 I.U. of regular insulin/kg, and 34 received Tolbutamide (2 or 3 g/kg). Insulin was injected subcutaneously and tolbutamide was given by gastric tube. Animals were killed or died in coma. *Group 2*: 84 adult rats weighing 180 to 440 g; 40 received insulin and 44 tolbutamide, at same doses and by same routes as in Group 1, and also killed or died in coma. *Group 3*: 26 adult rats weighing 200-400 g; 16 received Insulin and 10 received tolbutamide as in preceding groups, but these were injected intraperitoneally with 10 ml/kg of 10% glucose several times after administration of the hypoglycemic drug; these animals did not die in coma but were killed 5-8 hours after injection of hypoglycemic agent. B) Animals in which 2 to 5 hypoglycemic comas were induced. *Group 4*: 62

adult rats in which 2, 3, 4 or 5 insulinic comas were induced by same procedure, at intervals of 4 days between comas. Animals withdrawn from each coma by administration of 10 ml/kg 30% glucose given by gastric tube; 20 animals were killed or died during second, third, fourth or fifth coma, and 42 were sacrificed 1, 7, 18 and 34 days after recovery from each coma. C) Control groups: *Group 5*: 44 adult and 24 prepubertal rats which received saline solution by any route used for injection of insulin, tolbutamide or glucose. *Group 6*: 16 rats submitted to experimental conditions related to those suffered by groups 1 to 4, *i.e.*, 5 to 10 I.U. of ACTH, metrazolic convulsions (4 to 6 g), and barbituric comas (60 to 80 mg/kg).

All rats were fasted at least 12 hours before administration of hypoglycemic drugs. Glycemia was controlled in all groups by classical Somogyi-Nelson method. Testes were removed immediately after death of animal, and cut in slices not more than 1 mm wide. These slices were placed in Bouin, Zenker or Helly's fluids or in 10% formalin for fixation. For standard histological study 5 μ sections were colored with haematoxylin-eosin, and the following histochemical technics were also used: P.A.S. alsian blue and toluidin blue, (0.200 mg %, at pH 3.5.) Previous incubation with ptialin, hyaluronidase and methanol-chloroform was done as control for presence of substances to be detected by each of aforementioned histochemical stains, *i.e.*, glycogen, mucopolysaccharides and glycoproteins. Also Sudan IV was applied to study lipids.

Results. Rats in Groups 2 and 4 presented injuries, (72% and 100% respectively), in germinal epithelium, and congestion and oedema in interstitial spaces. No damage (0%) was seen in testis of prepubertal animals (Group 1) nor in control Groups 5 and 6; in this latter group only slight congestions were detected. Rats in Group 3, protected with glucose, which showed minimal degrees of hypoglycemia, also showed minimal (20%) or no injuries in their gonads.

Lesions of germinal epithelium consisted of: 1) sloughing, presumably originated in intercellular vacuoles (Fig. 3, 4, 5); 2) intracellular vacuolization of Sertoli cells, spermatogonia and spermatocytes (Fig. 3, 4); 3) Pyknosis of spermatogonia, spermatocytes, and, occasionally of spermatids (Fig. 6, 8); 4) Multinucleated masses of spermatocytes or spermatids (Fig. 6, 7).

Lesions 1 and 2 are present during and after first and second coma while Lesions 3 and 4 appeared in third, fourth and fifth coma. All these cellular alterations increased in number and intensity with repetition of comas. Not all types of lesions were present in each animal; some showed only one or 2 types of injury. The central tubes in a transverse section of glands were always those which showed more damage, and interstitial congestion and oedema were also more intense in this central zone. No lesions were observed in fibroblasts or in interstitial Leydig cells.

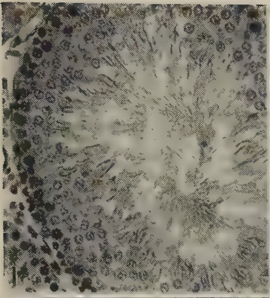
Rats which died with lowest levels of glycemia (under 30 mg %) bore more intense injury to their gonads. Group 3, only those animals in which level of glycemia was not completely protected by glucose showed some lesions.

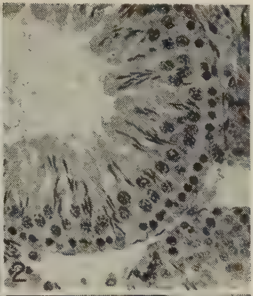
The rats from Group 4 killed at intervals from 1 to 34 days after last coma did not show complete recovery of testicular damage. Desquamative lesions disappeared in animals sacrificed between 7 and 18 days after second or third coma, but nuclear alterations and cellular vacuolization persisted 34 days after fourth and fifth coma.

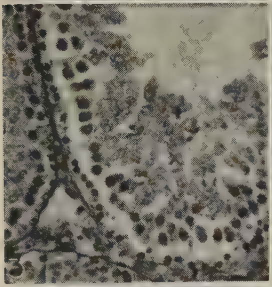
Histochemical technics showed negative results in all lesions studied. Intracellular and intercellular vacuoles did not show presence of glycogen, mucopolysaccharides, glycoproteins or lipids.

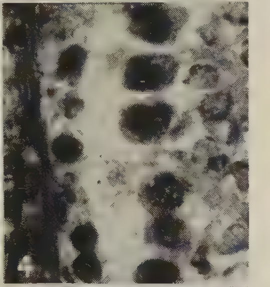
Discussion. Testicular injuries here described resemble description of encephalic injuries produced by hypoglycemia(2,3,4), also consisting of cellular vacuolization and pyknosis. In this sense the brain consumes glucose almost exclusively(1).

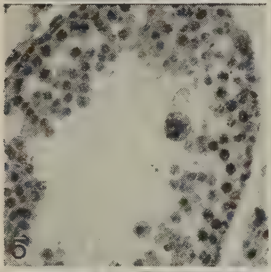
That lesions are more intense in animals with lowest glycemias, and that glucose could prevent very low glycemic levels, suggest that hypoglycemia is the specific factor responsible for them. Stress provoked by metabolic disturbance can be disregarded as a causative factor, because administration of ACTH did not produce detectable testicular damage in

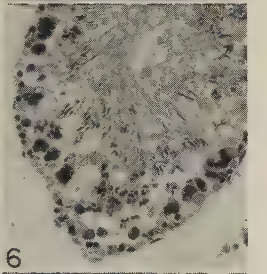


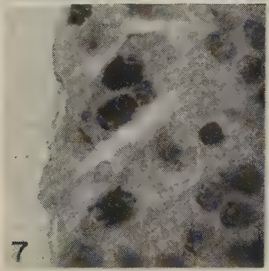












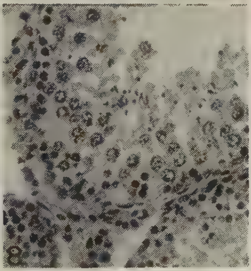


FIG. 1 and 2. Normal seminiferous tubules of adult rats in different stages of germinal epithelium. × 140 and 160 respectively.

FIG. 3. Seminiferous tubule of a rat which died in tolbutamide hypoglycemia. Note vacuolization and sloughing of germinal cells. × 140.

FIG. 4. Same case as Fig. 3. The higher magnification shows vacuoles in Sertoli cells and sloughing of spermatocytes. × 600.

FIG. 5. Seminiferous tubule of a rat killed during first coma. Sloughing has eliminated the layers of spermatids. Multinucleated masses being sloughed off. Intercellular vacuolization is intense. × 160.

FIG. 6. Tubule of rat which died in fifth coma. Note multinucleated cells and other nuclear abnormalities. × 140.

FIG. 7. Multinucleated masses in a tubule from a rat sacrificed at fourth coma. × 600.

animals of Group 6. Other authors proved that high doses of ACTH or corticoids in acute or chronic experiments did not show appreciable changes in the testis(5,6). The possibility that coma or convulsions were nonspecific factors causing testicular damage was also discarded; coma produced by Pentobarbital, and convulsions produced by Metrazol caused only slight interstitial oedema in testis of rats.

Tissue specificity of lesions seems high. No damage in cells, except interstitial congestion, was seen in liver or kidneys of rats bearing serious gonadal injury. This is also backed by lack of lesions in fibroblasts and Leydig cells in intertubular spaces. Injuries do not appear to be easily reversible; their intensity and persistence increased and were related to repetition of comas, and recovery is not complete, several weeks after last coma.

It is apparent that proliferation, differentiation, and greater metabolic turnover of germinal epithelium in adult animals as compared with prepubertal ones, renders them more liable to the deleterious effect of acute hypoglycemia.

Regarding histochemical nature of injuries, as vacuoles were negative to stains used, they may be temporarily ascribed to hydropic imbibition. The sequence of lesions revealed that these vacuolizations appear first and could be responsible for cellular sloughing. Nuclear abnormalities develop later and suggest severe disturbance in metabolism of nucleic acids and cell division. The significance of lack of glucose, which should deprive germinal cells of an important source of metabolic energy, is emphasized by our recent studies (unpublished) on oxygen consumption of rat testis using substrate with different carbohydrates. Also, it was shown that glucose is necessary to maintain male embryonic gonads incubated *in vitro*(7).

Whether injuries are secondary to interstitial oedema, we can only state that the latter was seen alone, with no accompanying tubular damage, but the reverse was not true.

FIG. 8. Rat killed during fifth coma. Injuries here are different as compared with Fig. 6. Pyknosis of nearly all spermatids is the main lesion here. × 160.

Summary. The histology of testis in albino rats submitted to hypoglycemic comas was studied. Comas were produced by either insulin or tolbutamide. No changes were seen in prepubertal animals, but in adults, injuries were seen in the seminiferous epithelium, coning of cellular and intercellular vacuoles, sloughing, nuclear pyknosis and multinucleated cells. Congestion and oedema were invariably present in the interstitium, though no injury was seen in any other testicular structure. Lesions increased in number and intensity with repetition of comas and recovery after comas was incomplete. Hypoglycemia is considered the specific cause of the injuries.

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Received June 16, 1960. P.S.E.B.M., 1960, v104.

Carbon Dioxide Fixation by Actinomyces . . . A Correction. (25958)

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Previously(1) I reported results of experiments on carbon dioxide fixation by strains of *Actinomyces* and *Lactobacillus* in which the data indicated a high incorporation of carbon dioxide into the methylene carbons of succinic acid. The data were supported by isolation of beta-alanine formed from succinic acid by the Schmidt degradation done at 40 to 70°C. Recently studies in this laboratory were initiated to determine the mechanism of this reaction in strains of *Actinomyces*. Although the initial experiments reproduced the previous results, isolation of the methylene carbons of succinic acid as ethylenediamine was preferred since it would eliminate several chromatographic steps and simplify the procedure. Although I had been unsuccessful previously in obtaining ethylenediamine by the procedure of Phares and Long(2) the use of a better grade fuming sulfuric acid and high temperatures permitted adequate formation of ethylenediamine. With some dismay, it was found that the carbon dioxide formed from the ethylenediamine was totally inactive. Subsequently, it was found that failure to adequately trap sulfur dioxide fumes formed by the reaction(3) accounted for the reduced specific activity in the carboxyl carbons. However, the close correlation of the specific activity of the isolated beta-alanine with that predicted is unexplained. The present results clearly show a net fixation of CO_2 into the carboxyl carbons of succinic acid. The remaining data published regarding fixation and formation of CO_2 are valid. It is, therefore, concluded that anaerobic fixation of CO_2 into succinic acid by *Actinomyces* primarily occurs *via* a Wood-Werkman reaction. In air, the oxidation of some intermediate of glucose metabolism results in formation of CO_2 and consequently reduces the specific activity found in the succinic acid.

I regret the error in the analyses and the concomitant circumstances which lead to a misinterpretation of the data. I would like to apologize for any inconvenience the use of such data may have caused.

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Received June 21, 1960. P.S.E.B.M., 1960, v104.

Hemopoietic Effects of Glyceryl Ethers. III. Inactivity of Selachyl Alcohol.* (25959)

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Prior to isolation of batyl alcohol from bovine yellow bone marrow by Holmes and associates(1), this ether of stearyl alcohol and glycerol had been thought to exist only in certain marine organisms. It has since been found in a number of mammals including man(2). Sandler(3) reported that batyl alcohol stimulated erythropoiesis in normal and benzene-poisoned rats. Two years later, Arturson and Lindbäck(4) described erythrocytosis and reticulocytosis in mice injected with this glyceryl ether. It has also been implicated in leukopoiesis(5,6) and thrombopoiesis (7). Our recent studies(8,9) confirmed the erythropoietic, thrombopoietic, and granulopoietic stimulatory activity of batyl alcohol. Although the exact physiologic significance of this compound is not known, the chemical and physiologic attributes common to batyl alcohol and the thermostable, ether-soluble plasma erythropoietic factor suggest that they may be closely related(10). However, many questions remain unanswered. Foremost among these is the apparent inconsistency between amount of batyl alcohol required to alter myelopoiesis in normal rats, *i.e.*, 25-50 mg per day(9), and the smaller quantities of a substance possessing specific biologic activity which would be expected to exert a readily detectable effect. Possible explanations include: 1) use of synthetic, racemic preparations rather than optically active, natural material or 2) close relationship to some other more active substance, *e.g.*, selachyl alcohol, $CH_3(CH_2)_7CH{:}CH(CH_2)_7CH_2O \cdot CH_2 \cdot CHOH \cdot CH_2OH$. The latter, which is the glyceryl ether found most abundantly in nature(11), differs only slightly in chemical structure from batyl alcohol, $CH_3(CH_2)_{16}CH_2O \cdot CH_2 \cdot CHOH \cdot CH_2OH$. However, selachyl alcohol is a liquid at room temperature, whereas batyl alcohol is a crystalline solid. This report describes experimental observations which bear directly on the above two possibilities.

Materials and methods. Forty normal female Wistar strain rats weighing 140-160 g were divided into 4 equal groups. The animals in each group received *via* gastric intubation 10 daily doses over a 2 week period (Saturdays and Sundays excepted) of one of the following test materials†: 1) 50 mg of synthetic, racemic selachyl alcohol; 2) 50 mg of optically active, natural selachyl alcohol; 3) 50 mg of optically active batyl alcohol prepared by hydrogenation of the natural selachyl alcohol and administered as a suspension in 0.5 ml of distilled water; or 4) 0.5 ml of distilled water. Baseline studies included hemoglobins determined by the cyanmethemoglobin technic, microhematocrits, hemacytometer erythrocyte and leukocyte counts, reticulocytes per 1000 erythrocytes enumerated on dried brilliant cresyl blue coverslip films counterstained with Wright's stain, and thrombocytes counted by phase microscopy. These measurements were repeated weekly. At end of treatment period, 5 rats in each group were killed and femoral marrow examined utilizing a technic previously described (12). Observations on the remaining animals provided information in respect to restoration of normal hemic equilibrium.

Results. The recipients of selachyl alcohol did not manifest evidence of altered hemopoiesis. Their peripheral erythroid values (Fig. 1) and thrombocyte and leukocyte counts (Fig. 2) remained stable. Marrow nucleated cell counts in these animals were comparable to those in the control group given water

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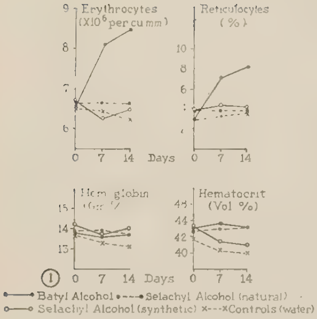
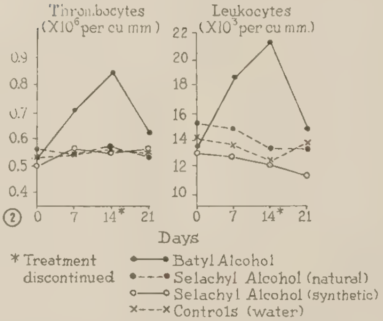
* Supported in part by Research Grant from N.I.H., U.S.P.H.S.

The technical assistance of M. C. Grayhack and N. E. Rosenberg is gratefully acknowledged.

† Batyl and selachyl alcohols used in this study were kindly supplied by Squibb Inst. for Med. Research, New Brunswick, N. J.





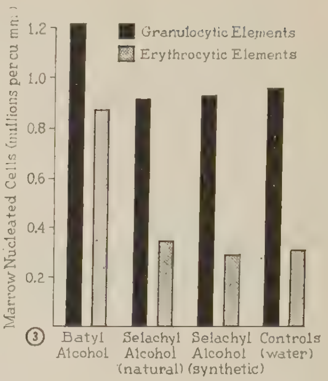


FIG. 1. Erythrocytosis and reticulocytosis without associated increases in hemoglobin or hematocrit levels in normal rats given 50 mg of batyl alcohol daily via gastric intubation. Recipients of comparable amounts of selachyl alcohol failed to manifest evidence of erythropoietic stimulation. Avg determinations of 10 animals receiving each of the above described test materials.

FIG. 2. Thrombocytosis and leukocytosis in normal rats given batyl alcohol. Normal values were restored in those animals not killed for marrow studies one wk after treatment was stopped. Thrombocyte and leukocyte counts remained constant in recipients of selachyl alcohol and were comparable to those obtained in the control group. Avg counts of 10 animals in each group.

FIG. 3. Myeloid erythrocytic and granulocytic hyperplasia in normal rats given 10 daily 50 mg doses of batyl alcohol *per os*. Megakaryocytes were also increased in these animals. Marrow nucleated cell counts in recipients of selachyl alcohol were similar to those obtained in the control group. Avg counts of 5 animals receiving each of the above described materials.

(Fig. 3). However, the batyl alcohol was fully active. The rats given this material developed erythrocytosis and reticulocytosis without associated increases in hemoglobin or hematocrit levels (Fig. 1), thrombocytosis and leukocytosis (Fig. 2), and increased numbers of both erythrocytic and granulocytic precursors in their marrows (Fig. 3). Type and magnitude of the responses were similar to those observed in other animals given racemic batyl alcohol(9). The microcytes responsible for the erythrocytosis were readily discernible and demonstrable by Price-Jones measurements. The leukocytosis was chiefly due to a relative and absolute neutrophilia. In those animals not killed for marrow studies, thrombocyte and leukocyte counts had returned to normal baseline levels one week after the treatment was stopped (Fig. 2). All animals remained healthy throughout experimental period, gained weight normally, and exhibited no adverse reactions to the test materials or repeated gastric intubations.

Batyl alcohol is active orally, and experimental data indicate that the glyceryl ethers can be absorbed intact from the gastrointestinal tract(13). However, the above experiment was repeated using a parenteral route of administration, *i.e.*, subcutaneous, to exclude with certainty any possibility of gastrointestinal inactivation. As before, each of the parameters selected to assess hemopoiesis remained unchanged in the recipients of selachyl alcohol.

Discussion. Despite the chemical relationship between batyl alcohol ($C_{21}H_{44}O_3$) and selachyl alcohol ($C_{21}H_{42}O_3$), the physiologic characteristics of these 2 substances differ markedly. Whereas selachyl alcohol was lacking in stimulatory activity under the above experimental conditions, comparable amounts of batyl alcohol, prepared by hydrogenation of the former, induced a clear-cut hemopoietic response in normal rats. These findings contribute greatly to the specificity of biologic activity of batyl alcohol. If the hematologic phenomena ascribed to batyl alcohol were nonspecific, *e.g.*, the result of an "irritative" or unphysiologic effect rather than primary myelopoietic stimulation, selachyl alcohol might be expected to possess similar properties. The possibility that selachyl alcohol may be an inactive precursor of batyl alcohol or some other substance has not yet been excluded.

It should be emphasized that the precise role which batyl alcohol may play in regulation of hemopoiesis is, as yet, conjectural. However, several points of interest have emerged from studies on hemopoietic effects of the glyceryl ethers and humoral control of blood cell production. Current data indicate that 2 humoral agents govern erythropoiesis (10). One substance, which seems to be a mucoprotein(14), stimulates hemoglobin formation most likely by augmenting erythrocytic differentiation of the pluripotential myeloid reticulum cells. Although maturation of erythrocytic precursors and synthesis of hemoglobin apparently proceed at fixed rates unaffected by the humoral stimulus, mitotic or proliferative activity of these marrow elements appears to be subject to a second humoral factor(10). The latter is thermostable, ether-soluble, and probably a lipid.

The singular type of erythropoietic response observed in normal rats given batyl alcohol is identical with that induced by the thermostable, ether-soluble plasma erythropoietic factor. It consists of erythromicrocytosis, reticulocytosis, and myeloid erythrocytic hyperplasia without associated increases in hemoglobins, hematocrits, or iron-59 uptake(9). In addition to this erythropoietic effect, the thermostable or ether-soluble fractions of plasmas from patients with polycythemia vera also induce, as does batyl alcohol, thrombocytosis and granulocytosis in normal recipient rats(15). Thus, there exist rather striking similarities between the chemical and physiologic properties of batyl alcohol and the thermostable, ether-soluble plasma factor which has been studied. Although persuasive of a close relationship, unequivocal support for this conclusion is not yet available.

The existent evidence is indirect and incomplete but is in accord with the thesis that batyl alcohol or some similar substance may play an important role in fundamental control of hemopoiesis. However, the answers to many problems must await further experimentation. For example, our studies fail to explain the need for the large amounts of batyl alcohol required to stimulate hemopoiesis in the normal rat. It may be simply a reflection of poor absorption and vastly different endogenous and exogenous requirements. It is hoped that additional observations on hemopoietic effects of the glyceryl ethers now in progress may help to elucidate the remarkable homeostatic mechanisms which govern hemopoiesis.

Summary. 1. Natural and synthetic selachyl alcohol is devoid of hemopoietic stimulatory activity in normal rats. 2. Batyl alcohol, derived from hydrogenation of selachyl alcohol, enhances proliferation of all myeloid elements but does not stimulate synthesis of hemoglobin. This effect is manifested in normal rat recipients by erythromicrocytosis, reticulocytosis, thrombocytosis, granulocytosis, and increased numbers of both erythrocytic and granulocytic elements in their marrows. 3. Batyl alcohol is active both orally and parenterally. The hemopoietic properties of this substance are not dependent on optical activity. 4. It is suggested, although not proved, that batyl alcohol may play an important role in regulatory control of hemopoiesis.

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Received June 22, 1960. P.S.E.B.M., 1960, v104.

Production of Acute Glomerulonephritis in Mice with Soluble Antigen-Antibody Complexes Prepared from Homologous Antibody.* (25960)

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Soluble antigen-antibody complexes, injected intravenously in antigen excess, are capable of producing the characteristic lesions of experimental serum sickness within a short period of time. When large amounts of either rabbit antiovalbumin-ovalbumin or rabbit anti-BSA-BSA (bovine serum albumin) complexes are injected into mice or rats a high incidence of acute glomerulonephritis results. In addition a focal arteritis and a valvulitis may be seen on occasion(1-4). Fluorescent labeled antibody technics have shown both the antigen and the antibody to be present at the site of the lesion. In the present study these observations have been extended to production of lesions with soluble complexes prepared from homologous mouse antibody. Antiovalbumin was produced in mouse ascitic fluid according to the method of Munoz(5) and compared with the corresponding rabbit antibody with regard to its ability to elicit glomerular lesions when administered to mice as soluble antigen-antibody complexes. The incidence and severity of anaphylaxis in both systems was studied. The effect of varying the antigen to antibody ratio in preparation of these complexes was also explored.

Materials and methods. Animals: Swiss Webster male and female mice weighing 20 to 30 g. *Antigens*: Hen ovalbumin, 3 times recrystallized, from Worthington Biochemical Co., Freehold, N. J. *Immunization*: Rabbit antisera against hen ovalbumin were prepared as previously described(3). Mice were immunized according to the method of Munoz. An emulsion containing 1 mg ovalbumin and 1 mg *Mycobacterium butyricum* per ml was prepared with Difco complete adjuvant. One ml was injected into the peritoneal cavity of each mouse on days 1, 18, and 38. Between 50 and 80% of the mice became ascitic, and between 1.0 and 15.0 cc of fluid was obtained from each animal. These collections were pooled. The various pools of antibody were analyzed for antibody protein content and antigen-antibody ratio at equivalence by the quantitative precipitin technic(6). Precipitates were analyzed for antibody protein by UV absorption at 280 mu in the case of rabbit antibody(7), and by the microKjeldahl method in the case of mouse antibody(6). Rabbit antiovalbumin. Pool I which contained 4.2 mg antibody protein/ml. Pool II which contained 4 mg antibody protein/ml. Mouse antiovalbumin Pool I which contained 8 mg antibody protein/ml. Pool II which contained 6.2 mg antibody protein/ml.

Preparation and injection of soluble complexes. Soluble complexes for injection into mice for the experiments on glomerulonephritis were prepared by direct addition of excess antigen to either the rabbit antisera or mouse ascitic fluid at room temperature. Volume was adjusted to 0.5 ml per injection and injected into the tail vein within 5 minutes. For the studies on anaphylaxis soluble complexes were prepared as previously described, by dissolving the washed immune precipitate in antigen excess(3). *Preparation of histolo-*

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* Aided by grants from U. S. P. H. S. and from Kidney Disease Fn. of N. Y.

TABLE I. Incidence and Severity of Glomerulonephritis in Mice Given 3 Injections of Soluble Antigen-Antibody Complexes in 24 Hr, and Killed 24 Hr after 3rd Injection.

Material inj.	No. of mice	No. of mice with glomerulonephritis Mild	Moderate	Severe	Total
Soluble complexes*	12	3	4	5	12
Antibody alone	10	0	0	0	0
Antigen "	3	0	0	0	0
Normal mice	33	0	0	0	0

* 2, 3, and 3 mg mouse antiovalbumin antibody protein/inj. as soluble complexes in 5 times antigen excess.

gic sections. The mice were sacrificed by decapitation 24 hours after last injection and tissues were fixed in 10% buffered formalin. Sections of heart, lung, liver, spleen, and kidney were routinely stained with hematoxylin and eosin.

Results. A preliminary series of experiments showed that doses of one mg or more of mouse antibody protein in the form of soluble complexes in 10 times antigen excess, produced a mortality from anaphylaxis of nearly 100%. To reduce incidence of anaphylactic deaths, animals used in the studies on glomerulonephritis were first given 0.25 mg of antibody protein in 5 times antigen excess one to 2 hours prior to administration of the larger doses. Animals so treated suffered no mortality from subsequent injections.

A group of 12 mice were given a series of 3 injections of mouse antiovalbumin in the form of soluble complexes in 5 times excess antigen. Each mouse received 2 mg antibody protein in the first injection and 3 mg in each of the 2 subsequent injections, all within 24 hours. In addition 3 animals were given antigen alone and 10 were given antibody alone in the same doses.

Incidence and severity of the lesions observed in these animals are shown in Table I. Glomerulonephritis was present in all of the experimental animals.

The nature of the glomerular lesion was similar in all of the animals. Both kidneys were uniformly involved. The glomeruli were hypercellular, had swollen cells, were relatively bloodless, and were infiltrated with neutrophiles; in severe cases karyorrhexis was present (Fig. 2). In many of the animals a striking accumulation of amorphous, eosinophilic material was seen in the glomerular capillary loops (Fig. 3).

Two animals showed a mild acute inflammation of the mitral valve. These lesions were similar to those previously described(3).

Since the intensity of the glomerular lesions was equal to or greater than that previously seen when rabbit antibody was used, an attempt was made to compare the two systems and to determine minimal effective dose of mouse antiovalbumin antibody necessary to produce renal lesions.

Two groups of 12 mice each were given a series of 3 injections of soluble complexes in 24 hours. The first group received 1 mg of antibody protein per injection as ovalbumin-rabbit antiovalbumin in 5 times antigen excess. The second group received exactly the same schedule of treatment, but mouse antiovalbumin was used instead of the rabbit antibody. A third group of 6 animals were given mouse antibody in the same manner as the second group but received only 0.25 mg antibody protein/injection. A fourth group of 3 mice were given a single injection of 2.0 mg antibody protein in the form of ovalbumin-mouse antiovalbumin complexes in 5 times antigen excess. These last animals were survivors from an anaphylaxis experiment described below, and did not receive previous in-

TABLE II. Comparative Ability of Soluble Antigen-Antibody Complexes Prepared from Mouse and Rabbit Antiovalbumin to Produce Acute Glomerulonephritis in Mice.

Soluble complexes made with	No. of mice	No. of mice with glomerulonephritis Mild	Moderate	Severe	Total
Rabbit antibody 1 mg × 3*	12	4	0	0	4
Mouse antibody:					
1. 1 mg × 3*	12	5	5	1	11
2. .25 mg × 3†	6	0	0	0	0
3. 2 mg × 1‡	3	2	1	0	3

* 1 mg antiovalbumin protein/inj., 3 inj., complexes in 5 times antigen excess.

† .25 mg antibody protein/inj., in 10 times antigen excess.

‡ One inj. of 2 mg antibody protein, in 5 times antigen excess.

jections. Incidence and severity of the lesions in the 4 groups of mice are indicated in Table II.

It is apparent from the results presented that soluble complexes prepared with mouse antibody are more effective than those prepared with rabbit antibody in producing glomerulonephritis in mice. Furthermore a single injection containing as little as 2 mg of mouse antibody protein in 5 times antigen excess can produce mild acute glomerulonephritis within 24 hours.

Since administration of soluble antigen-antibody complexes is invariably followed by anaphylaxis, the relative abilities of the mouse and rabbit systems to elicit the reaction was investigated. The effect of varying ratio of antigen to antibody on occurrence of anaphylaxis was also studied. The experimental data are shown in Table III.

As in production of experimental glomerulonephritis, mouse antibody also proved to be much more effective than rabbit antibody in producing anaphylaxis. In both systems, a decreased incidence of anaphylaxis was observed when either antigen to antibody ratio or dose of antibody protein was reduced.

Discussion. Intravenous administration of soluble antigen-antibody complexes to mice, using homologous antibody in the form of immune ascitic fluid, results in a very high incidence of severe acute glomerulonephritis. The lesions are more severe than those produced with equivalent amounts of rabbit antibody. The greater pathogenicity of complexes prepared with mouse antibody is further emphasized by their ability to produce the characteristic pathologic changes within 24 hours after a single injection containing 2 mg antibody protein.

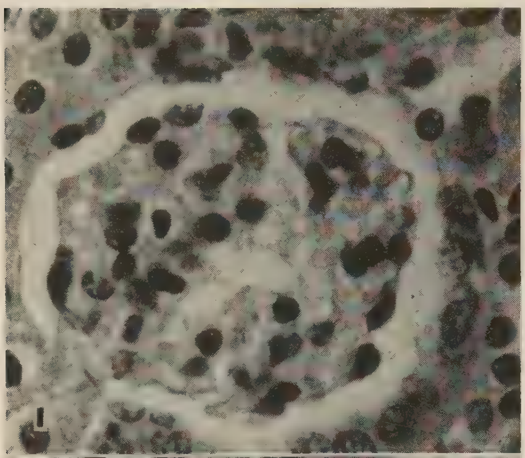

FIG. 1. Kidney of normal mouse. Hematoxylin and eosin stain. ×880.

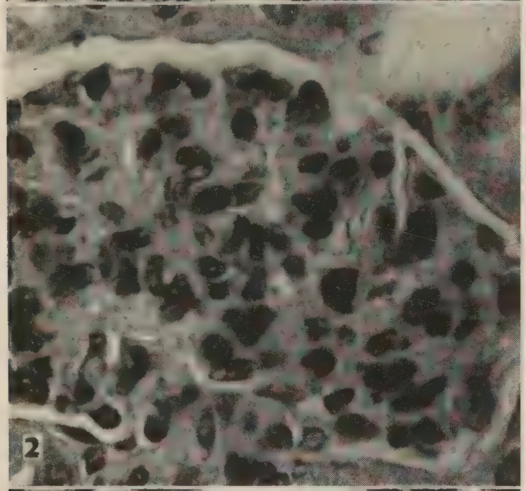

FIG. 2. Kidney of a mouse given 3 inj. of ovalbumin-mouse antiovalbumin complexes, 2, 3, and 3 mg antibody protein per inj. respectively, and sacrificed 24 hr later. Glomerulus is hypercellular, bloodless, and infiltrated with neutrophiles. Hematoxylin and eosin stain. ×880.

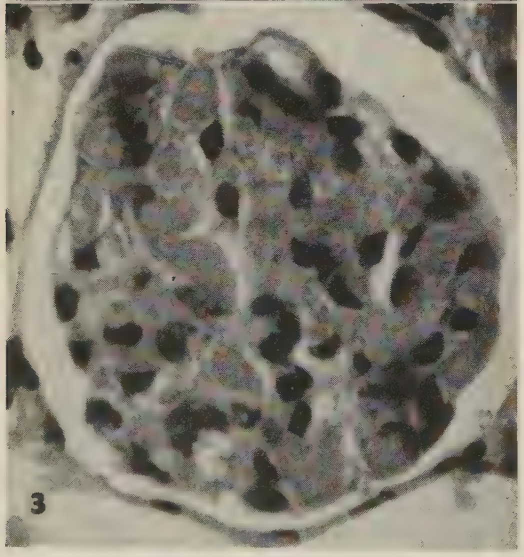

FIG. 3. Kidney of a mouse given 3 inj. of ovalbumin-mouse antiovalbumin complexes, 2, 3, and 3 mg antibody protein per inj. respectively, and sacrificed 24 hr later. A striking accumulation of amorphous, eosinophilic material is seen in glomerular capillary loops. Hematoxylin and eosin stain. ×880.

TABLE III. Effect of Varying Ratio of Antigen to Antibody and Dose of Antibody on Production of Anaphylaxis: A comparison of rabbit and mouse systems in the mouse.

Antibody protein (mg)	Antigen excess (times)	Mouse antiovalbumin		Rabbit antiovalbumin	
		No. mice	No. deaths	No. mice	No. deaths
.25	5	5	0	5	0
	10	5	2	5	0
	20	5	3	5	0
	50	5	3	5	0
1	5	15	11	16	4
	10	18	14	5	2
	20	4	3	5	3
	50	6	6	11	8
2	5	8	5	5	3
	10	4	3	5	3
	20	2	1	5	5
	50	—	—	5	5

The appearance of the glomeruli was essentially the same in both systems except for the marked accumulation of amorphous eosinophilic material in capillary loops of those animals given complexes prepared with homologous antibody. Similar findings had been previously observed in animals given soluble rabbit complexes following pretreatment with cortisone(3). In those animals this material was shown to contain both antigen and antibody by the Coons' technic and was presumed to be precipitated complexes. In the present experiments these findings may be explained by the low levels of antigen excess used. Since ovalbumin is normally excreted by kidneys of mice, use of only slight excess antigen would favor aggregation of the circulating soluble complexes in the glomeruli.

Finally, the observations on occurrence of anaphylaxis in mice treated with soluble complexes prepared with mouse antiovalbumin in comparison with rabbit antiovalbumin indicate that homologous is considerably more effective than heterologous antibody. The data concerning variations of antigen to antibody ratios and dosage of antibody are substantially in accord with those previously reported by other investigators(8,9).

Summary. 1) Passive serum sickness was produced in mice using homologous antibodies, obtained from immune ascitic fluid, in the form of soluble antigen-antibody complexes. Lesions were similar to those obtained with rabbit antibody-antigen complexes, but, in addition, amorphous eosinophilic material was seen in many glomerular capillary loops. Incidence and intensity of lesions using homologous antibody was greater than with equal amounts of heterologous rabbit antibody. 2) Homologous antibody was more effective in producing lethal anaphylaxis than rabbit antibody when both were given as soluble complexes.

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Received June 24, 1960. P.S.E.B.M., 1960, v104.

Stimulation of Erythrocyte Metabolism by Menadione.* (25961)

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The human erythrocyte in the presence of an autoxidizable dye such as methylene blue can metabolize glucose *via* the hexose monophosphate shunt. In fact, as much as 85% of oxygen consumed by the erythrocyte can be accounted for by this mechanism(1). Since the demonstration(2,3) that respiration of the erythrocyte can be stimulated by methylene blue, investigators have sought a physiological electron carrier to substitute for the dye. Most of these attempts have met with little success, although it has been reported that a toxoflavin from *Pseudomonas cocoven* stimulates the enzyme system(4). Substances reported to be without effect are riboflavin, flavin adenine dinucleotide, reduced glutathione, ascorbic acid and ergothionine(3). The present study indicates that menadione will substitute for the artificial carrier in the erythrocyte system and stimulate oxygen consumption by the cells. It is further demonstrated that such stimulation is dicumarol sensitive and can be appreciably decreased when dicumarol is present in the reaction flasks.

Methods. Blood collected in acid citrate-dextrose was centrifuged to remove the buffy coat and to collect the cells. These were subsequently washed with additional acid citrate-dextrose followed by 3 washings with Krebs-Ringer phosphate buffer pH 7.4. The cells were finally resuspended in the buffer, volume of which was adjusted to give a final hematocrit of 50. Adenosine (10 μmoles/ml) was added to all preparations(3). Oxygen uptake was determined by the conventional Warburg technic. Coenzyme Q_{10}† and α-tocopherylquinone were added to the reaction in bovine plasma albumin, ethanol, buffer suspension, 5 mg coenzyme Q_{10} or α-tocopherylquinone, 2 mg albumin, 0.1 ml ethanol per ml suspension.

Results. Table I summarizes the results obtained when the erythrocytes were incubated with menadione. Upon incubation, oxygen consumption of the cells compared favorably with that obtained with methylene blue or other autoxidizable dyes. When concentration of menadione was varied, oxygen consumption also varied. For example, menadione (.15 μmole) caused an oxygen consumption of 50.9 μl/hr/hematocrit of 50; increasing the concentration to 0.31 μmole resulted in an oxygen consumption of 76.7 μl/hr/hematocrit of 50.

Since it has been demonstrated that dicumarol is an effective inhibitor of Vit. K(5), it was felt to be of interest to assess its role in the menadione stimulated erythrocyte system. Addition of dicumarol to the system resulted in inhibition of oxygen consumption produced by menadione (Table II). On the other hand, dicumarol had no effect when methylene blue was used to stimulate the system.

The fact that menadione, a napthoquinone, was capable of maintaining oxygen consumption by the erythrocyte prompted evaluation of like compounds in this study. Coenzyme Q_{10} and α-tocopherylquinone were without effect.

Other compounds capable of acting in redox systems were also tried. The following were without effect: ergothionine, dehydro-ascorbic

TABLE I. Effect of Menadione on Q_{O_2} of Human Erythrocytes.

Additions (μmole)		Q_{O_2}, μl/hr/hematocrit of 50 Mean	Range
Methylene blue	(.31)	79.5	71.1-88.4
Menadione	(.31)	76.7	72.6-82.5
”	(.15)	50.9	48.7-55.0
”	(.05)	14.1	12.8-15.2
”	(.03)	6.0	4.5-10.6

Each Warburg flask contained: 1 ml erythrocytes, 30 mmoles glucose, 1 ml buffer pH 7.4, 0.2 ml 6 N NaOH. Menadione or methylene blue added as indicated. Total vol 3.2 ml. Oxygen uptake values corrected for endogenous respiration.

Menadione was dissolved in 7% alcohol prior to use. Alcohol controls exerted a negligible effect on the system.

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* Supported in part by Nebraska Fund for Psychiatric Research.

† Kindly supplied by Merck, Sharp & Dohme.

TABLE II. Effect of Dicumarol on Erythrocyte System.

	% inhibition	
	Menadione	Methylene blue*
Dicumarol, μmoles	(.31 μmole)	
.31	25.9	0
1.5	44.0	0
3.1	50.1	0

System same as indicated under Table I. Dicumarol added at the expense of buffer.

* A slight potentiation of action of methylene blue was noted with dicumarol which appeared to be independent of dicumarol concentration.

acid, hypoxanthine, and oxidized glutathione.

Discussion. The results presented here would suggest that in addition to autoxidizable dyes, menadione is capable of supporting oxygen consumption in the erythrocyte system. It is tentatively assumed that oxygen uptake in the presence of menadione, like that in the presence of methylene blue, is a measure of the glucose oxidative pathway in this tissue since, in the human erythrocyte, oxygen utilization occurs almost entirely through the hexose monophosphate shunt. Similar results have been reported(6) on a rat liver fraction which reoxidizes TPNH (reduced triphosphopyridine nucleotide), where it was noted that the enzyme preparation reacted with electron acceptors, notably Vit. K and coenzyme Q_{10}, but the diaphorase was particularly active in presence of Vit. K_3 (menadione).

The sensitivity of the menadione stimulated system to dicumarol and the lack of effect with dicumarol in presence of methylene blue implies that the effects noted with dicumarol are specific for menadione and do not necessarily imply interference with the action of the erythrocyte system.

Menadione has been previously demonstrated to be effective in increasing the activity of the phosphogluconate pathway in ascites tumor cells(7). Subsequent studies(8) also noted the dicumarol inhibitory effect on C-1 oxidation *via* the pentose pathway. However, the present experiments are the first demonstration of such an effect in the human erythrocyte.

Of added importance to this study is the nature of the defect in congenital methemoglobinemia (Type I). Since this deficiency can be corrected by methylene blue, it would be of interest to know if menadione would substitute for methylene blue in the treatment. This would appear to be a reasonable possibility because both compounds are most likely serving in the electron transport system between reduced pyridine nucleotides and methemoglobin.

It is not immediately apparent why coenzyme Q_{10} and α-tocopherylquinone did not support oxidation by the pentose pathway. The high concentration of alcohol required to solubilize these compounds may have inactivated the system; also, the side chain on these compounds may have prevented access to sites within the cell necessary for activity. Of interest also is the fact that addition of CoQ_{10} to ascites cells failed to stimulate decarboxylation *via* the phosphogluconate oxidative pathway(8). Studies are now in progress on isolation of the enzyme system and the role of coenzyme Q_{10} and like substances will be evaluated on the isolated system.

Summary. In the human erythrocyte, menadione is capable of supporting oxygen consumption. In this respect, it can replace autoxidizable dyes such as methylene blue that have been previously used. Oxygen consumption, as a measure of the pentose pathway, is inhibited by dicumarol.

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Received June 27, 1960. P.S.E.B.M., 1960, v104.

Serum Leucine Aminopeptidase in Twin Pregnancy.[*][†] (25962)

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Since the original report(1) describing colorimetric determination of leucine aminopeptidase activity (LAP) in human sera using L-leucyl-β-naphthylamide hydrochloride as a synthetic substrate, many observers(2,3,4,5,) have recorded elevated levels of this enzyme in the sera of pregnant human females. Published results are in general agreement in showing a gradual rise in serum levels of LAP values throughout the second and third trimesters of pregnancy, with the amount of this enzyme at term being from 2 to several times the amount present in the normal non-pregnant female or in the pregnant female during the first 12 weeks of pregnancy. It has been reported(3) that in 3 cases of hydatidiform mole and one case of choriocarcinoma LAP activity of the serum was normal, and it has been stated(4) that serum levels of this enzyme in patients with pre-eclampsia or chronic hypertensive disease were found to be the same as in normal pregnancy. No data are yet available on serum LAP activity in multiple gestation, therefore we report the following series of patients with documented twin pregnancies.

Material and methods. Sera were obtained, during the third trimester, from 11 patients with radiographically proven twin pregnancies, and from 25 patients during the same stage of gestation with single fetuses. Serum LAP activity was determined by hydrolysis of L-leucyl-β-naphthylamide hydrochloride, with a colorimetric assay of the β-naphthylamine liberated. A .2 ml sample of serum is diluted with 9.8 ml of distilled water and a 1 ml aliquot of this mixture is incubated with 0.18 mg of L-leucyl-β-naphthylamide hydrochloride dissolved in 2 ml of a 0.18 M phosphate buffer at pH 7.2. Incubation is for 2 hours at 37°C. Hydrolysis is then stopped with 1 ml of 40% trichloracetic acid and 1 ml of 0.1% sodium nitrite is added to a 1 ml sample of the mixture in a colorimetric tube. Three minutes later, after diazotization is complete, excess sodium nitrite is decomposed with 1 ml of 0.5% ammonium sulfamate. A blue color is then produced by addition of 2 ml of N-(1-naphthyl) ethylenediamine dihydrochloride in 95% ethanol (0.5 mg per ml) and this is read against a blank on the spectrophotometer at 560 mμ. Values are recorded as optical density x 10^3.

TABLE I. Comparison of Serum Leucine Aminopeptidase (LAP) Activity[*] in Single and Twin Pregnancy in the Third Trimester.

Pregnancy	No. of cases	Wk gestation	Mean values and S.D.	Range
Twin	11	31-43	1072 ± 84	920-1230
Single	25	31-41	618 ±128	347- 860

[*] Values recorded as optical density × 10^3.

Results are presented in Table I. Comparison of the 11 patients with proven twin pregnancies with the control group representing the same stage of gestation (third trimester) reveals a significant difference. Using the t test of significance for non-paired samples, t = 12.7***, which exceeds the 0.1% level of significance.

LAP activity of amniotic fluid was found to be 50 units on 2 determinations.

Discussion. The etiology of the increase of serum LAP activity in single and twin pregnancy as well as in cancer and other diseases is unknown. Umbilical cord blood shows low levels of activity as do sera from patients with trophoblastic tumors(3). Tissue analyses of this enzyme in human placentas reveal no significant increase in LAP activity per unit of weight of placenta as gestation progresses(4).

Since the placental mass in twin pregnancy is usually greater than the placenta of a single fetus, our results would suggest that the amount of normally functioning placental tissue is related to LAP activity in the maternal serum.

Summary. Serum leucine aminopeptidase

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[*] This work supported by U.S.P.H.S. research grant RG 4679(C3).

[†] We acknowledge the help of Dr. Gilbert J. Vosburgh during early phases of this work.

(LAP) activity during third trimester of pregnancy was significantly increased in 11 patients with documented twin pregnancy, when compared with 25 patients with a single fetus of same gestational age.

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Received June 20, 1960. P.S.E.B.M., 1960, v104.

Biological Assay of Human Urinary Erythropoietic Stimulating Factor (ESF)* (25963)

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Previous studies have demonstrated the presence of ESF in urine of patients with a variety of anemias(1-4). Because of the ready availability of Thalassemia patients with predictably low levels of hemoglobin our efforts have been restricted to these subjects. An attempt has been made to correlate concentration of ESF in the urine with hemoglobin level in the blood of donor subjects at the time urine was collected. Although the data obtained previously were insufficient to indicate any association between these factors(4), the evidence now available is sufficient to suggest that concentration of ESF in urine does relate to severity of anemia. This information is useful as a guide in selection of patients as donors of ESF. Based upon the hematocrit and reticulocyte responses to ESF, a bioassay has been developed which is probably more reliable than existing ones which depend upon the use of only one parameter in evaluating erythropoietic responses.

Materials and methods. Thirty-four samples of urine were collected from 13 patients with Thalassemia major at hemoglobin levels ranging from 2.5% to 8.0%. The samples were frozen immediately and subsequently thawed, filtered, and frozen in sterile injection bottles until assayed. Mature 170 to 230 g Long-Evans female rats, maintained upon a definite surplus supply of water and Purina Laboratory Chow for at least 2 weeks after removal from the stock cages, were used for assessment of erythropoietic effects. The size of the groups used varied from 3 to 5 animals depending upon the precision required and availability of material. Each rat received 5 daily 3 ml subcutaneous injections of urine or extract. Microcapillary hematocrit and reticulocyte values were determined prior to assay, and upon the day after last injection. Reticulocytes were stained with new methylene blue according to the procedure of Brecher *et al.*(5). With the aid of an ocular counting disk, one-fourth of the erythrocytes in a microscopic field were counted while all reticulocytes in the field were enumerated and divided by 4. The percent reticulocytes was calculated after counting 1000 cells.

Results. In Fig. 1 and 2, reticulocyte and hematocrit responses are plotted separately as functions of hemoglobin levels of donor patients. Two equations were derived from the analysis of these relations by the method of least squares(6). The first equation describes the regression of reticulocyte responses in recipient rats (y) upon hemoglobin levels of donor patients (x).

$$\text{(a)}\quad y = -2.1x + 13.2$$

The second equation represents the regression of hematocrit responses in recipients (z) upon donor hemoglobin levels (x).

$$\text{(b)}\quad z = -1.8x + 11.1$$

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* Supported by Grant, Nat. Inst. Health, PHS.

† From thesis submitted in partial fulfillment for degree of Ph.D.

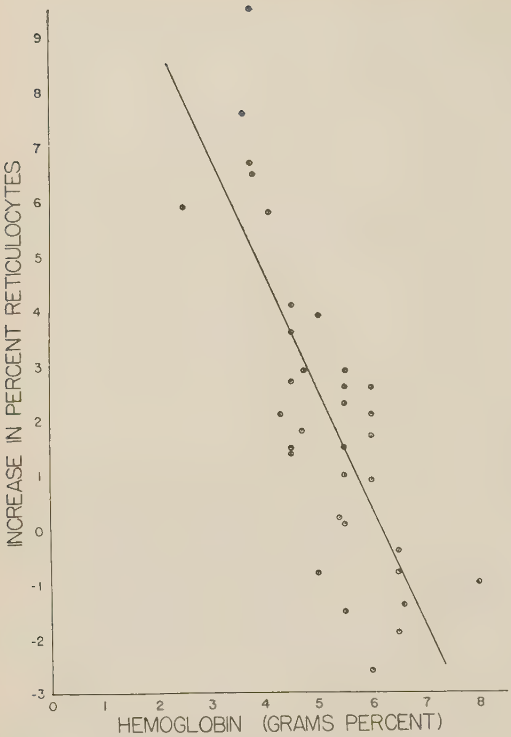

FIG. 1. Reticulocyte responses in ESF treated rats as a function of hemoglobin values of donor patients.

It is clear from the negative slopes of these regression lines that ESF concentration in urine increases with decreasing hemoglobin levels in the donor patients. When the 2 equations are solved simultaneously, an expression for y as a function of z is obtained:

(c) $y = -1.16z + .03$

Since the y intercept in relation (c) is small, it may be disregarded, and because the slope 1.16 within the limits of scatter of the data is approximately equal to 1, equation (c) may be simplified to $y = z$. With this convenient approximation, hematocrit and reticulocyte changes (obtained on sixth day after initiation of the injections only) may be regarded as equivalent and can therefore be averaged or added. The value obtained by addition of the rise in hematocrit percentage to the increase in reticulocyte percentage is referred to as the H-R unit. Potency of an ESF preparation is thus expressed in terms of response in H-R units obtained by injecting a stated daily dose in mg or ml for 5 days.

In Fig. 3, response to a single preparation is plotted as a function of urine dose in ml. The response is neither a linear nor a logarithmic function of the dose. Nevertheless, comparisons may be made between preparations diluted to an activity of about 10 H-R units. In some cases, approximate comparisons may be made between preparations assaying from 0-10 H-R units with the assumption that the response is nearly linear in that range. By comparison of the response in terms of H-R units to a standard preparation (CS-1) supplied by Dr. Henry Borsook with the response to this preparation estimated by the method of Fried *et al.*(8) and White *et al.*(9), it has been tentatively established that a daily dose which produces a 1.3 cobalt unit response by the latter method will elicit an H-R response of 1 unit.

Discussion. It is established that urinary concentration of ESF increases with severity of anemia in Thalassemia subjects. The existence of this relation serves as additional evidence favoring the view that ESF is a feed-

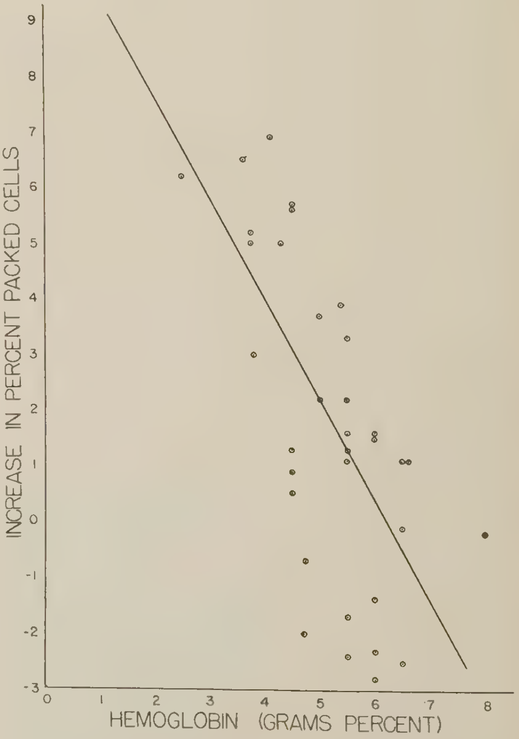

FIG. 2. Hematocrit responses in ESF treated rats as a function of hemoglobin values of donor patients.

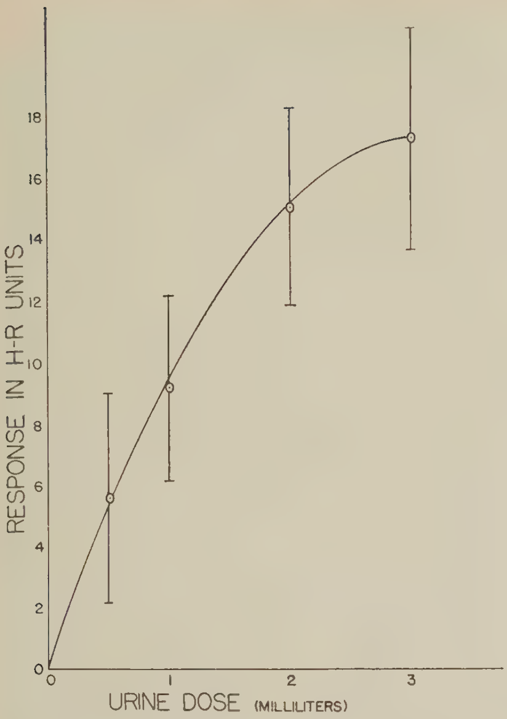

FIG. 3. Responses of rats in H-R units as a function of daily doses of ESF expressed in ml of urine. The line is drawn through the mean values for the difference between increases in H-R units observed in 3 treated and 3 untreated control rats. Height of the vertical lines above and below mean values indicates stand. errors of differences in means.

back agent which is present in greater concentration when there is a greater demand for increased erythropoiesis. From the curves in Fig. 1 and 2 it may be noted that a high potency urine from Thalassemia subjects can reasonably be expected when their hemoglobin levels fall below 4 g %. The cut-off point for appearance of ESF in urine is approximately at hemoglobin levels of 6 g %. It is not certain whether this cut-off point occurs at the renal threshold, or is merely a fall in ESF concentration to levels which are not detectable by the assay. A similar drop in ESF in plasma to undetectable levels has been noted by Lowy *et al.* working with rabbits at equivalent low hemoglobin values(7). It may be that severe anemia enhances the discrepancy between rate of production and rate of utilization of ESF such that the factor accumulates to demonstrable levels.

The biological assay for ESF in terms of a single parameter such as the reticulocyte response alone, or Fe^{59} uptake by red cells(8,9) is hazardous since any agent which produces a hemolytic anemia would be likely to produce a positive response. Use of 2 parameters, such as the reticulocyte and hematocrit responses tends to remove this difficulty but it would appear cumbersome to base a unit on 2 separate assay parameters. Nevertheless, it has been found possible to add reticulocyte and hematocrit increments (in 6-day assays only) and thus obtain a single H-R unit. This unit has the advantage that hemolytic effects frequently observed in animals responding to crude urine are likely to be cancelled out. Once the potency of a crude urine sample is established in terms of H-R units, fractionation of ESF is more economically followed by the method of Fried *et al.*(8) and White *et al.*(9) since their assay, which is based on the Fe^{59} incorporation into the erythrocytes of starved rats, requires injection of less ESF. On the other hand, for screening large numbers of samples of urine for ESF activity under circumstances where only a few samples are likely to be potent, the H-R unit assay is the more economical one, for it eliminates the costs involved in use and disposal of Fe^{59}.

Summary. Concentration of ESF in the urine of Thalassemia patients increases with severity of anemia. Subjects with hemoglobin levels below 4 g % may reasonably be expected to provide urine with a high titre of human ESF. A biological assay depending upon the single unit obtained by the additive changes in hematocrit and reticulocyte values of rats has been developed which produces a cheaper and more reliable method of screening samples for ESF activity than the starved rat red cell Fe^{59} uptake procedure. The latter method, however, is more sensitive and therefore more useful in following the purification of ESF preparations of established potency.

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Received July 1, 1960. P.S.E.B.M., 1960, v104.

Effect of Dietary Lipids on Growth of Transplantable Neoplasms in Mice.[*] (25964)

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The effects of total restriction of diet on incidence and growth of neoplasms in experimental animals have been summarized by Tannenbaum(1) and by Cowdry(2). Neoplasms inhibited by dietary restriction are spontaneous mammary tumors, induced skin tumors, induced sarcomas, primary lung tumors, and leukemias. Spontaneous tumors are affected more than are artificially induced tumors.

Diets with a high content of fat have enhanced development of certain spontaneous and artificially induced neoplasms in rats and in mice(3), including stilbesterol-induced mammary carcinoma in rats, chemically induced hepatic carcinoma in rats, and carcinoma of the skin in mice produced by tars and polycyclic hydrocarbons. The fatty acid composition and degree of saturation of the fats used in most of these experiments varied and showed no consistent effect, but varied with individual neoplasms.

This report deals with a comparison of the effects of diets containing various lipids upon incidence and rate of growth of L1210 lymphatic leukemia in DBA/2 mice and the adenocarcinoma 755 in C57 Bl/6 mice(4). These transplantable tumors were selected because their characteristics are consistent and predictable. The dietary fats were selected to compare the effects of fats with a high content of unsaturated fatty acids with those with a lower content of unsaturated fatty acids.

Materials and methods. L1210 leukemia in DBA/2 mice. Two hundred fifty adult DBA/2 mice, 125 males and 125 females, were divided into 5 dietary groups of 50 mice each, (25 males and 25 females). They were approximately 4 months of age when special diets were substituted for *Purina Laboratory Chow*. The artificial diets were identical in composition except for fat content (Table I). The basic material, "fat-free" diet powder[†], consisted of vitamin-free casein (21.1%), alphacel "cellulose" (16.45%), sucrose (58.45%), salt mixture (4%), and vitamins. Diet A (group A mice) consisted of the basic powder, Vit. B_{12} (3 μg/100 g diet), folic acid (0.2 mg/100 g diet), and ascorbic acid (0.1 g/100 g diet). Diets B, C, and D (groups B, C, and D) consisted of the basic diet powder and added vitamins plus 11% (by weight) of corn oil,[‡] hydrogenated vegetable oils,[§] or lard.[‖] The characteristics of these various fats are shown in Table II.

The mice were maintained on the special

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[*] Supported by grant from U.S.P.H.S.

[†] Nutritional Biochemicals Corp., Cleveland.

[‡] Mazola, Corn Products Co., Argo, Ill. furnished through the courtesy of Mr. Neal E. Artz.

[§] Shortening J (similar to Crisco), Procter and Gamble Co., Cincinnati, furnished through the courtesy of Mr. W. H. Meyer.

[‖] Reliable Packing Co., Chicago, Ill., through the courtesy of Mr. Hugo E. Wistreich, who also supplied a chemical assay of this product.

TABLE I. Composition of Synthetic Diets.

	"Fat-free" base			Fat			% total calories, final diet mixture			
	Protein (casein)	Carbohydrate (sucrose)	Cellulose and salts	Corn oil	Hydrogenated vegetable oils	Lard	Fat	Carbohydrate	Protein	Total calories/100 g final diet mixture
	g/100 g diet									
A	21.1	58.5	20.5					73.5	26.5	358
B	18.8	52.3	18.3	10.5			22.9	56.5	20.5	415
C	18.8	52.3	18.3		10.5		22.9	56.5	20.5	415
D	18.8	52.3	18.3			10.5	22.9	56.5	20.5	415

diets, *ad lib.*, and tap water, for 7 months prior to inoculation with L1210 leukemia. During this preliminary period, mice from each group expired from various causes. Several mice of group A (minimal fat diet) developed signs of "essential fatty acid deficiency" (5), in most instances promptly relieved by addition of minimal amounts of corn oil to the diets.

Seven months after the beginning of the special diets, half of the surviving mice in each group received, intraperitoneally, 0.1 ml of a 1:100 dilution (in 0.25% methyl cellulose in 0.85% NaCl) of ascitic fluid that had been freshly aspirated from the peritoneal cavity of a DBA/2 mouse with L1210 leukemia (approximately 71,000,000 tumor cells per mouse). The mice which were not inoculated served as controls.

The mice in all groups, both inoculated and uninoculated, continued to receive the special diets, *ad lib.*, and tap water, until spontaneous deaths occurred. Daily total body weights of all inoculated mice and periodic body weights of uninoculated control mice were made.

All of the L1210-inoculated mice expired within 12 days following inoculation (Fig. 1). Fluid was withdrawn from the peritoneal cavity of these mice, smeared on slides and stained with Wright's stain. Histologic sections were made of organs from all mice.

Adenocarcinoma 755 in C57 Bl/6 mice. Eighty male C57 Bl/6 mice (age 4 months) were divided into 4 dietary groups of 20 each. Groups A, B, and C received diets identical in composition except for the fat content and tap water, *ad lib.* Group A received diet A, (fat-free), group B received diet B (11% corn oil), and group C received diet D (11% lard). Group D (controls) received *Purina Laboratory Chow.* The composition of these diets was identical to those described in the preceding section.

Five days after beginning of the special diets, 10 mice in each group received a subcutaneous implant of fresh adenocarcinoma 755 which had been removed from the donor mouse and chopped into uniform pieces 1 to 2 mm in diameter. To eliminate variation in viability of the tumor cells, implants were given alternately to the mice in each group. The 40 control mice received no tumor.

All mice, including the uninoculated controls, continued to receive the special diets. The inoculated mice were examined daily for tumors, which, in the majority, appeared within 8 or 9 days as a subcutaneous nodule

TABLE II. Characteristics of Fats.

		Predominant constituent fatty acids in %								
		Saturated					Unsaturated			
Fat	Iodine No.	Myristic	Palmitic	Palmatitic	Stearic	Approx. total	Oleic	Linoleic	Linolenic	Approx. total
Corn oil	125	.2	9.9		2.9	13.0	30.1	56.2		86.3
Hydrogenated vegetable oil product*	76	Saturated fatty acids consist primarily of palmitic and stearic acids.				24.5	65	10	.5	75.5
Lard	59 (approx.)	1.9	29.5	3.1	16.5	51.0	37.0	9.4		46.4

* Shortening J consists primarily of soybean and cottonseed oils.

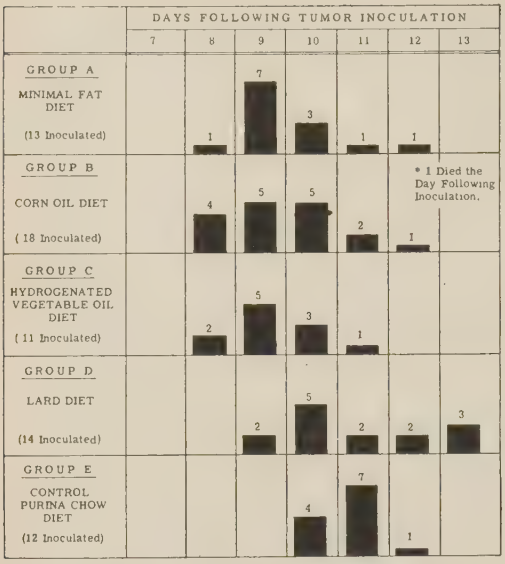

FIG. 1. Survival of DBA/2 mice after inoculation on day 1 with L1210 leukemia. Numbers over vertical bars represent No. of animals.

near site of inoculation. Total body weights of all mice were determined on alternate days. The tumors were measured daily by means of a vernier caliper and consisted of the product of the greatest dimension and a dimension at a 90 degree plane from the greatest. Several tumors grew rapidly resulting in death of the hosts. Because by the 21st day, a total of 6 mice had expired, surviving mice of all groups, including uninoculated controls, were sacrificed by deep chloroform anesthesia. The tumors were carefully removed and weighed in the fresh state. Because metastases were found in only 2 mice, they were not included in the final tumor weights. Histologic sections were made of the organs and tumors from all mice.

Results. L1210 leukemia. Examination of smears of the peritoneal fluid and histologic sections revealed that all inoculated mice had developed classical lymphatic leukemia. There was no significant difference in distribution of leukemic infiltration in any of the dietary groups. All mice had slight to moderate infiltration of the liver; more than one-half had definite involvement of the bone marrow (sternum), and adrenal glands. Insignificant numbers of mice in various groups had infiltration of the lungs, epicardium, kidneys, skin, and gastrointestinal tract.

None of the uninoculated control mice developed signs of leukemia during the interim between inoculation of L1210 leukemia and death of the inoculated mice, nor did any die during this period. A few are living after 17 months of maintenance on the special diets; some have ultimately developed spontaneous lymphatic leukemia or other neoplasms. The results of the effects of the various diets upon development of the spontaneous neoplasms will be reported later.

Adenocarcinoma 755. There was no significant difference in the mean time of appearance of tumors in any of the 4 dietary groups (Table III). By the 9th day after tumor implantation, one-half of the mice in each group had grossly palpable tumors; by the 21st day all inoculated mice in all groups had detectable tumors. *None of the uninoculated controls developed tumors.* All of the 6 mice (one in group A, 2 in group B and 3 in group D) which had expired by the 21st day had large tumors. Fig. 2 shows rate of growth of the tumors in each dietary group expressed as the product of 2 dimensions as described above.

Tumors of mice of all groups grew at approximately the same rate until the 14th day when those of group B mice began to lag slightly. The fastest rate of tumor growth occurred in groups A and D. Tumor weights compared favorably with measurements calculated in this manner (Table IV). The largest tumors occurred in group D (controls, regular diet) and the smallest in group B (artificial diet with corn oil). Some of the tumors were not weighed because portions had

TABLE III. Time of Appearance of Grossly Palpable Tumors.*

Dietary groups of mice	Days after implantation 10	13	15	22
A	5	8	8	10
B	5	9	10	10
C	5	8	8	10
D	6	8	8	9†

* Figures in vertical columns represent total No. of mice with grossly palpable tumors on any day.

† One mouse expired on day of tumor implantation.

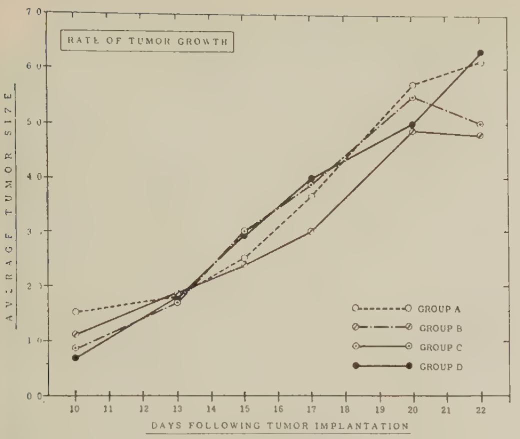

FIG. 2. Rate of growth of adenocarcinoma 755 in C57 Bl/6 mice. Tumor size is expressed by product of 2 dimensions (in cm), as explained in text.

become necrotic and in several instances had been partially eaten by other mice.

Although the mice of group A received no fat during the 4 weeks of the experiment, none developed overt signs of "essential fatty acid deficiency"(5), nor did the lack of fat significantly affect development or rate of growth of the tumors. Incidence and rate of growth of tumors in mice of all dietary groups were almost identical to those of the mice maintained on *Purina Laboratory Chow* (approximately 5% fat). Average total body weights of the uninoculated control mice on diets with 11% (by weight) of fat were not more than one or 2 grams higher than those of the mice receiving *Purina Laboratory Chow*. The mice which developed tumors appeared to gain weight, but when final tumor weights were subtracted from total body weights, it was found that they actually lost weight as the tumors developed.

TABLE IV. Comparison of Weights and "Surface Areas" of Tumors on Day of Death.

Dietary groups of mice	Wt of tumors (g)		"Surface areas" of tumors*	
	Avg	Range	Avg	Range
A	5.03	.91-8.64	4.8	.1-9.4
B	3.70	1.35-5.90	4.8	3.1-7.8
C	4.73	.18-9.20	5.0	.5-8.5
D	6.29	4.46-7.99	6.3	5.4-8.7

* Calculated by method described in text.

Histologic sections of inoculated mice revealed that all had typical adenocarcinomas. Metastatic tumors were found in only one mouse of group A and one of group C, in both cases to the liver and abdominal lymph nodes. *Uninoculated mice did not develop neoplasms of any type.*

Summary. The incidence of susceptibility of inbred mice to 2 transplantable neoplasms, L1210 leukemia and adenocarcinoma 755, was not influenced by variation in the dietary fats. Diets deficient in fat likewise had neither inhibiting nor stimulating activity in the pathogenesis of the 2 neoplasms.

The authors are indebted to Dr. Clyde Culbertson and Dr. Irving Johnson, Eli Lilly and Co., for help in supplying mice and transplantable tumors, and to Mr. James F. Glore, Dept. of Illustration at the Medical Center, for preparation of graphs. The capable technical assistance of Pearl Driver, Betty Corya, Shirley Stockslager, and Donna King is also appreciated.

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Received June 30, 1960. P.S.E.B.M., 1960, v104.

Testosterone Regulation of 17β-Hydroxysteroid Dehydrogenases.* (25965)

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Guinea pig liver and kidney utilize diphosphopyridine nucleotide (DPN) or triphosphopyridine nucleotide (TPN) as the hydrogen acceptor in conversion of testosterone to 4 - androstene - 4,17 - dione(1,2). The coenzymes are linked with specific enzymes which function independently even in an homogenate(3). This report indicates that the activity of these enzymes in the kidney but not the liver is regulated by testosterone.

Methods and materials. DPN (Pabst) was used as purchased. Testosterone† for enzyme assay was further purified(4) and dissolved in water at 32 μg/ml(3). Testosterone propionate‡ was injected subcutaneously daily. Normal and control guinea pigs were injected with sesame oil. *Animals.* Guinea pigs (Zeimet Bio-Farms) were kept in screen bottom metal cages in an air conditioned room at 22-23°. They were fed Jim Dandy rabbit pellets with a supplement of 50 mg of ascorbic acid twice per week (Table I) or Rockland guinea pig diet with supplements of 30 g carrots and 25 mg ascorbic acid twice per week (Table II). Castration was performed under ether anesthesia. Guinea pigs were stunned by blow at base of skull and bled by severing blood vessels at neck. Liver and kidneys were removed and homogenized in ice-cold 0.25 M sucrose. Liver homogenate was diluted to 5 g/100 ml and the kidney to 10 g/100 ml. Dehydrogenases were determined as previously described(3). The reaction mixture contained: 1.0 ml 0.18 M sodium pyrophosphate pH 9.5, 1.8 ml testosterone 32 μg/ml and 0.2 ml of DPN (15 mg/ml) or TPN (5 mg/ml). Water was substituted for testosterone in the blank cell. The reactants were warmed to 40° prior to use. The reaction was initiated by addition of the ice-cold homogenate in the following amounts: DPN enzyme 0.06 ml liver, 0.15 ml kidney and TPN enzyme 0.08 ml liver, 0.05 ml kidney. The appearance of reduced coenzyme was recorded at 340 mμ in a Cary Model 14 Spectrophotometer at 38°. A unit of enzyme has been designated as a change in optical density of 0.001 per minute.

Results. Body and organ weights. Changes in body and organ weights after castration and testosterone propionate treatment were as expected(5,6,7). The androgen produced a small increase in body weight and restored the weight of seminal vesicles and prostates (Tables I, II). Liver and kidney weights were not significantly changed by either castration or testosterone propionate injections.

17β-hydroxysteroid dehydrogenases. Activity of enzymes of the liver was not significantly affected by castration or androgen treatment (Table I). Enzymes of the kidney, on the other hand, were decreased by castration and restored to normal by injections of androgens for 14 days. Similar results were obtained when doses of 5, 10 and 25 mg/day of testosterone propionate were administered for 65 days (Table II). Restoration of activity of the dehydrogenases preceded restoration of weight of the accessory sex organs (Tables I, II).

Discussion. The difference in response to castration and androgen treatment between the 17β-hydroxysteroid dehydrogenases of the kidney and liver suggest that these enzymes are associated with different functions in the 2 tissues. Many enzymes which apparently catalyse identical reactions have been demonstrated to respond differently in liver and kidney after castration or androgen administration(8). Furthermore, enzymes of the kidney have proved to be more responsive to concentration of androgen in the body(8,9). It is of interest, therefore, that the 17β-hydroxysteroid dehydrogenases of the liver were not affected while those of the kidney responded to castration and androgen administration.

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* These studies were aided by research grant from Nat. Cancer Inst. P.H.S.

† Testosterone provided by Syntex.

‡ Testosterone propionate provided as Perandren by Ciba Pharmaceutical Products.

TABLE I. Effect of Castration and Testosterone Propionate on DPN and TPN 17β-Hydroxysteroid Dehydrogenases of Liver and Kidney of Male Guinea Pig.*

		17β-Hydroxysteroid dehydrogenases			
		Liver		Kidney	
	Sem. ves. & pros., g	DPN	TPN	DPN	TPN
		u/mg			
Normal	3.92	10.5	5.5	.94 ± .083	4.0 ± .27
Castrated†	.57	10.5	6.2	.65 ± .035	2.5 ± .14
” † + T P‡	2.54	10.3	5.9	.86 ± .063	4.0 ± .19

* Seven animals/group.

† Guinea pigs castrated at 58 days of age, 406-553 g body wt. Injections begun 36 days later and continued 13 to 16 days.

‡ Testosterone propionate (Perandren, 50 mg/ml) was inj. subcut. at 25 mg/day.

It should also be noted that administration of the androgen restored activities of the 2 enzymes to normal but not above normal level. Responses of the dehydrogenases are similar to those observed for arginase and alkaline phosphatase(9).

The presence and relative activity of the steroid dehydrogenases have been suggested as regulator mechanisms for the manifold effects of androgens and as an explanation for their differential effect on various tissues and among animal species(1,8). The guinea pig apparently uses 17β-hydroxysteroid dehydrogenases of the kidney and not the liver as a regulator to maintain a homeostatic mixture of androgens in its body. The doses of androgen were excessive and prolonged, yet accessory sex organs and muscles(7) were not stimulated to greater than normal levels. Localization of this mechanism in the kidney might be peculiar to the guinea pig. Administration of androgens alters activity of the 3β and 3α-hydroxysteroid dehydrogenases in the liver of the rat and other species but not that of the guinea pig(10). The adrenal cortical steroids also influence dehydrogenases of rat-liver. Administration of prednisone acetate or prednisolone acetate increased the activity of an adrenal cortical steroid dehydrogenase (change in Porter Silber reaction)(11). This increase in enzyme activity has been suggested as the mechanism for increased tolerance of glucocorticoids by patients after prolonged treatment with these steroids.

TABLE II. Effect of Dose and Extension of Testosterone Propionate Injections on 17β-Hydroxysteroid Dehydrogenase Activities of Guinea Pig Liver and Kidney.*

		17β-Hydroxysteroid dehydrogenases			
		Liver		Kidney	
	Sem. ves.† & pros., g	DPN	TPN	DPN	TPN
		u/mg			
Normal	6.49	9.5	5.1	1.09	3.5
Castrated	1.05	8.6	5.5	.77	2.3
Castrated‡ + 5 mg/day TP§	5.53	8.5‖	5.5‖	1.14	3.6
Castrated‡ + 10 mg/day TP§	6.04	10.5¶	5.1¶	1.07	3.9
Castrated‡ + 25 mg/day TP§	7.16	9.2	5.4	1.09	4.0

* Four animals/group.

† Kidney and liver wt not significantly changed.

‡ Castration performed at 68 days of age 549-698 g body wt.

§ Inj. begun day of castration and continued daily except Sunday for 65 days. Daily dose was contained in 0.5 ml of sesame oil.

‖ Single determination.

¶ Two determinations.

Summary. Castration of male guinea pigs produced a decrease in activities of DPN and TPN 17β-hydroxysteroid dehydrogenases of the kidneys. Subcutaneous injection of testosterone propionate for 14 days restored activities of kidney enzymes. Extension of injections to 65 days gave no further changes. Dehydrogenases of liver were not affected by castration or androgen administration. Ratio of DPN to TPN dehydrogenase was approximately 2:1 in liver and 1:4 in kidney. DPN enzyme activity of liver was approximately 10 times that of kidney.

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Received June 17, 1960. P.S.E.B.M., 1960, v104.

Erythrocyte Repopulation in X-Irradiated Recipients of Nucleated, Peripheral Blood Cells of Normal Mice. (25966)

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Unitarian and polyphyletic theories of blood cell formation have been discussed for many years(1). Many hematologists agree that the fixed stem cell may be pluripotent, but the capacity of more mature forms, and particularly of circulating cells, to dedifferentiate toward less-mature blast forms is not universally accepted. Studies with tissue culture technics have indicated that numerous morphological types can be derived from apparently pure blood cell cultures(2). Much information about blood cell differentiation may also be obtained by use of an *in vivo* culture system in mice, in which normal hematopoiesis has been destroyed by X radiation. In such mice, Merwin(3) showed that leukocytes of leukemoid blood produce immunologically competent cells, and Smith *et al.*(4) reported that leukemoid leukocytes differentiated into erythroblasts. Since leukemoid blood contains many immature cells, preliminary observations that circulating leukocytes from normal mice may produce erythroblasts in irradiated recipients are herein reported because of their important implications about the dynamics of normal hematopoiesis.

Materials and methods. A program was initiated to produce coisogenic strains of mice that differ at the hemoglobin locus. The scheme was essentially similar to that of Snell (5). The "single" type hemoglobin of strain C57BL mice was genetically introduced in strain BALB/c, RF, AKR, and 101 mice, which possess "diffuse" type hemoglobins, and the "diffuse" hemoglobin of 101 mice was introduced into C57BL mice. Technics of starch gel electrophoresis(6) and hemoglobin solubility(7) were used to identify mice that carried the introduced, genetically controlled, type of hemoglobin. At present, the coisogenic status of these strains varies from 5 to 8 generations of backcrossing.

Sixteen-week-old BC_5 coisogenic 101 mice, which were progeny of the BC_4 backcross to normal 101 mice and were heterozygous for diffuse and single hemoglobins, were given 600 r of X rays and used as recipients. X ray conditions were 250 kvp, 31.5 ma, hvl 0.43 mm of Cu; the dose rate in air was approximately 80 r/min, tissue to source distance was 93.7 cm. Donor mice were anesthetized with Nembutal, ventral skin was removed, and the sternum was excised to expose the heart. Cardiac blood was withdrawn by means of a hypodermic needle and syringe that contained 2% sodium citrate as anticoagulant. Twelve to 15 ml of blood was pooled from 10 normal 101 donors, 3-4 months old. White blood cells were separated in 15-ml centrifuge tubes by centrifugation at 600 X *g* for 15 minutes. The buffy coat and many underlying red cells were removed with a pipette and placed in Wintrobe hematocrit tubes to be centrifuged a second time at 600 X *g* for 20 minutes. Ap-

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* Operated by Union Carbide Corp. for U. S. Atomic Energy Commission.

TABLE I. Percentage of Donor-Type Erythrocytes.

Exp.	Animal	Days after treatment 15	29	46	49	63
I	1	0		0		
	2	30		60*		
II	3		†		40	25
	4		0		0	0
	5		60		100	100

* Mouse died on day 69.

† Analysis indicated that the graft was partially successful, but the mouse was very anemic and quantity of donor-type erythrocytes cannot be determined accurately under such conditions.

proximately 100 million nucleated, buffy coat cells were injected intravenously *via* the tail vein into the 600 r X-irradiated recipients. Microscopic examination of stained preparations showed that the buffy coat contained many platelets and all forms of normal circulating white blood cells. Aggregation of white blood cells was prevented by use of 2% sodium citrate. The technic of HbCO solubility(7) was used to determine the percentage of donor-type erythrocytes in irradiated recipients 30-60 days after treatment.

Results. Five X-irradiated, coisogenic BC_5 101 mice, heterozygous for diffuse and single hemoglobins, have been injected with ~ 100 million buffy coat cells removed from normal 101 mice. The percentage of donor-type red cells derived from the injected white cell mixture is indicated in Table I for each recipient.

Discussion. Although none of the circulating cells of normal blood are classified as erythroblasts, the data suggest that cells that separate with the white blood cell fraction can differentiate into erythropoietic cells. Similarly, no leukemoid blood cells are classified as erythroblasts, but leukemoid leukocytes do incorporate tritium-labeled thymidine *in vitro* and do differentiate into erythroblasts *in vivo* (4).

Recipient mice in these experiments are not completely coisogenic; nevertheless, the infusion of white blood cells was performed in a parent-to-F_1 manner, *i.e.*, parental white blood cells to partially coisogenic recipients, so that the tendency for rejection of the graft at midlethal doses of X rays was minimized (8). Analyses made 15-30 days after infusion of white blood cells may have been somewhat affected by presence of donor-type erythrocytes in the white cell fraction injected; however, analyses at 60 days should not have been affected by contamination of donor red cells since the life span of mouse red blood cells is reported to be approximately 60 days(9). Furthermore, increasing quantities of donor-type red cells in succeeding tests indicate that grafted cells must have been actively proliferating. The strain 101 mice used in this particular experiment were supplied by a commercial breeder, and it has recently been discovered that his 101 stock was not isogenic (C. C. Congdon, personal communication); perhaps the failure of transplantation in mice Nos. 1 and 4, and graft regression between days 49 and 63 in mouse No. 3 (Table I), resulted from graft rejection. The proportion of donor-type rbc in the latter is greater than what might have occurred without at least transitory production of new cells by grafted progenitors.

Data presented support the unitarian theory of blood cell formation in that they suggest that circulating cells are capable of differentiating into erythroblasts. Perhaps one of the morphological types we classify as fairly mature (*i.e.*, the monocyte or large lymphocyte) is really a free stem cell capable of differentiation into many cell types. Bond *et al.* (10) showed that a small number of such cells in peripheral blood of normal human beings incorporate tritium-labeled thymidine and consequently presumably can divide. From studies of blood cultures, Maximow(11) and Downey(12) concluded that a distinction between the lymphocyte and hemocytoblast is perhaps artificial. Furthermore, Pierce(13) and Downey and Stasney(14) showed that staining characteristics of a cell do not clearly indicate its maturity since mature cells may have more basophilic cytoplasm than less-mature forms. Whether our results can be ascribed to the lymphocytic, granulocytic, or erythrocytic series, or perhaps all of these, is being investigated by using tissues primarily composed of such elements and peritoneal exudates.

The adoptive transfer of transplantation immunity conferred by use of whole blood

(15) and the deleterious effect of isologous whole blood for homologous bone marrow transplantation in mice(16) should be reinterpreted in the light of these findings. Transplantation immunity can be conferred with as few as 50,000 cells of a Peyer's patch(17) and 80,000 isologous bone marrow cells are able to protect against hematopoietic failure induced by X radiation(8). Hence, since the white blood cell count is ~ 8 million per ml of whole blood, the proportion of the circulating cells that are able to divide may be as high as 1%.

Summary. Strains of mice are being produced that are isogenic with inbred stocks except for the hemoglobin locus. With mice of such strains, experiments were carried out to ascertain whether peripheral blood of normal mice contain cells capable of repopulating the marrow of irradiated recipients. In 3 of 5 recipients injected intravenously with ~ 100 million nucleated peripheral blood cells after 600 r, increasing numbers of graft-derived erythrocytes were noted within 60 days after treatment, indicating the presence in peripheral blood of cells that are able to give rise to mature erythrocytes.

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Received June 29, 1960. P.S.E.B.M., 1960, v104.

A Study of the So-Called "Cat Effect" of Cruickshank and Whitfield. (25967)

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Some years ago Cruickshank and Whitfield (1) claimed on rather scant evidence from time-concentration curves on cats that "gobbling" of T-1824 by the reticulo-endothelial system leads to an overestimate of plasma volume upon first injection of this dye. Furthermore, they suggested that "a previous 'saturating' dose of dye should be injected before a determination is made in order to abolish the 'absorption curve' ". Judging from the questions directed to our laboratory from time to time, even as recently as the present year, we have the impression that Cruickshank and Whitfield's report, often referred to as the "cat effect," is still considered unchallenged. To be sure, the experiments of Cruickshank and Whitfield have not, to the best of our knowledge, been exactly repeated but since the question has been raised with us again, we decided to report the results of experiments made by one of us in 1946-47 (E.N.) which were designed to test the "cat effect." Our reasoning was that if the claims regarding this phenomenon were true, then the plasma volume obtained by the second of 2 successive injections of T-1824 should be smaller than the first. In a series of experiments on unanesthetized normal cats and dogs, in which 2 successive determinations of plasma volume were made with T-1824, we found that the outcome was quite the opposite.

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Materials and methods. The experiments were carried out on 11 dogs and 9 cats without anesthesia after a preliminary period of training. The T-1824 injections were made into the jugular vein with a calibrated syringe immediately after withdrawing the usual control blood sample. In dogs the average dose of T-1824 for each test was 11.5 mg/kg body weight and in cats, 9 mg/kg. The post injection blood samples were drawn from the contralateral veins at 5, 10, 15, 20, 30, and sometimes also at 45 and 60 minutes after each injection of dye and immediately transferred to hematocrit tubes containing an adequate amount of powdered heparin to prevent clotting. The second injection was made immediately after drawing the last sample from the first series and subsequent dye-tinged samples were read against this "blank," the first series being read against the initial dye-free control sample, either in a Koenig-Martins spectrophotometer at 620 millimicrons (cats) or with a Nickerson decade photometer (dogs). Optical densities were then plotted on semilog paper and extrapolated in the usual manner to determine D_o for calculating plasma volume. Plasma protein concentrations were measured with a refractometer and from these the dye curves were corrected for fluid shifts to obtain fair estimates of the dye disappearance rates. Hematocrit values obtained by centrifugation at 3000 rpm (radius 19 cm) for 30 minutes were corrected for plasma trapping using the factor 0.96. It should be noted that total blood volumes were calculated using the equation $BV = \frac{PV \times 100}{100 - Hct \times 0.96}$. Hence the total blood volume values have not been adjusted for the F_{cells} factor which in any event is known to be subject to large variations in the unanesthetized dog(2) and perhaps also in the cat.

Results. The relevant data from the experiments on cats and dogs are shown in Tables I and II respectively; mean values and standard deviations are given at the bottom of each table. In all but one experiment on cats (4M where the 2 plasma volumes were the same) the second plasma volume was larger than the first, the mean difference for all 9 experiments being 8.4% (s.d. ± 4.6). In the dogs the second plasma volume was also larger than the first except in 6F where the second volume was nearly 3% less than the first. However, we may note that in this experiment neither hematocrit value nor plasma protein concentration showed the gradual fall characteristic of the other tests and usually observed with repeated sampling. Nevertheless, in the dogs the second plasma volume averaged 6.4% (s.d. ± 7.8) larger than the first, corresponding fairly well with the difference noted in the cat experiments.

Disappearance rates of T-1824 are shown at extreme right of each table. These are higher in cats than in dogs and also higher after the first injection than after the second in both groups of animals. Although heart rates reveal fairly large individual variations in both cats and dogs, it will be seen that in any one experiment the values did not change radically during the period of the test.

Plasma volumes in ml/kg body weight in the cats ranged from about 30 to 56 with a mean value of 41 (± 8). In the dogs corresponding values range from about 45 to 83 with a mean of 63 (± 14). Total blood volumes in cats ranged from about 52 to 97 ml/kg with a mean of 71 (± 16); in dogs the range was from 78 to 145 with a mean of 110 (± 20). As stated above, these total blood volumes cannot be taken as true values since the calculations were made without regard for unequal distribution of red cells in the circulation which in any event is unpredictable in conscious dogs(2) and perhaps also in cats with the spleen intact.

Discussion. The fact that the disappearance rates are higher after the first than after the second injection of dye would seem to lend support to the claims of Cruickshank and Whitfield, *but only if the first plasma volume were larger than the second.* We have seen from Tables I and II that quite the reverse

TABLE I. Normal Unanesthetized Cats.*

Cat No. and sex	Date	Wt	Hct × .96, %	Plasma protein, % (refract)	Heart rate/min.	Plasma vol, ml	Plasma vol, ml/kg	Dev. of 2nd plasma vol from 1st plasma vol, %	Blood vol, ml	Blood vol, ml/kg	Disappearance rate, %/hr
1 ♂	11/19/46	4.0	1) 38.5 2) 36.5 3) 36.0	1) 7.98 2) 7.74 3) 7.32	1) 208 2) 209 3) 200	1) 146 2) 153	1) 36.5 2) 38.3	+ 4.9	1) 243 2) 254	1) 60.8 2) 63.5	1) 15.6 2) 11.7
2 ♂	6/20/46	3.3	1) 37.5 2) 35.2 3) 32.9	1) 7.00 2) 6.45 3) 5.98	1) 230 2) 232 3) 232	1) 100 2) 109	1) 30.8 2) 33.6	+ 9.1	1) 170 2) 182	1) 52.3 2) 56.0	1) 31.0 2) 16.1
3 ♀	11/26/46	3.0	1) 42.8 2) 42.0 3) 40.3	1) 7.32 2) 7.32 3) 7.22	1) 208 2) 184 3) 168	1) 120 2) 142	1) 40.0 2) 47.0	+17.5	1) 218 2) 252	1) 73.0 2) 84.0	1) 30.6 2) 15.3
4 ♂	6/12/46	3.6	1) 42.7 2) 41.1 3) 39.2	1) 7.52 2) 7.46 3) 7.01	1) 164 2) 148 3) 142	1) 202 2) 202	1) 56.2 2) 56.2	0	1) 357 2) 350	1) 99.1 2) 97.3	1) 22.2 2) 20.0
5 ♂	6/13/46	3.1	1) 42.6 2) 40.8 3) 41.7	1) 7.32 2) 6.96 3) 6.54	1) 210 2) 220 3) 232	1) 125 2) 135	1) 40.4 2) 43.4	+ 7.4	1) 227 2) 246	1) 73.2 2) 79.4	1) 13.9 2) 14.3
6 ♀ (preg.)	12/12/46	2.5	1) 44.5 2) 41.7 3) 41.0	1) 7.88 2) 7.30 3) 7.22	1) 220 2) 232 3) 248	1) 89.5 2) 97.5	1) 35.8 2) 39.0	+ 8.9	1) 166 2) 178	1) 66.4 2) 71.1	1) 26.0 2) 20.4
7 ♀	1/ 6/47	2.5	1) 36.8 2) 35.0 3) 34.4	1) 6.60 2) 6.19 3) 5.62	1) 222 2) 225 3) 232	1) 80 2) 85	1) 32 2) 34	+ 6.3	1) 135 2) 141	1) 54.0 2) 56.5	1) 39.2 2) 23.8
8 ♀	12/ 5/46	3.3	1) 28.2 2) 29.3 3) 28.4	1) 7.11 2) 7.11 3) 6.65	1) 190 2) 208 3) 192	1) 149 2) 170	1) 45 2) 51.5	+14.4	1) 218 2) 250	1) 66.4 2) 75.7	1) 34.9 2) 19.0
9 ♀	12/ 6/46	2.6	1) 44.8 2) 44.7 3) 45.4	1) 6.65 2) 6.65 3) 6.65	1) 152 2) 152 3) 176	1) 134 2) 148	1) 51.5 2) 57.0	+12.6	1) 252 2) 280	1) 97 2) 108	1) 40.0 2) 18.2
10 ♀	12/ 2/46	1.8	1) 35.2 2) 34.2 3) 32.2	1) 7.74 2) 7.57 3) 7.00	1) 240 2) 220 3) 188	1) 75.0 2) 76.9	1) 41.6 2) 42.7	+ 2.6	1) 127 2) 122	1) 70.5 2) 67.8	1) 40.8 2) 20.8
Mean		2.97	1) 39.4 2) 38.1 3) 37.3	1) 7.31 2) 7.08 3) 6.72		1) 123 2) 132	1) 41.0 ±8.1 s.d. 2) 44.3 ±8.5 s.d.	+ 8.4 ±4.6 s.d.	1) 211.3 2) 225.5	1) 71.3 ±15.8 s.d. 2) 75.9 ±16.9 s.d.	1) 29.4 2) 17.9

* For Hct, plasma protein and heart rate: 1) time of first dye inj.; 2) time of second dye inj.; 3) end of second dye curve. For plasma vol, blood vol and disappearance rate: 1) from first dye curve; 2) from second dye curve.

TABLE II. Normal Unanesthetized Dogs.*

Dog No. and sex	Date	Wt, kg	Hct × .96, %	Plasma protein, % (refract)	Heart rate/min.	Plasma vol, ml	Plasma vol, ml/kg	Dev. of 2nd plasma vol from 1st plasma vol, %	Blood vol, ml	Blood vol, ml/kg	Disappearance rate, %/hr
1 ♂	4/29/46	11.0	1) 43.5 2) 41.6 3) 41.3	1) 6.01 2) 5.86 3) 5.50	1) 60 2) 64 3) 63	1) 553 2) 560	1) 50.3 2) 50.7	+ .80	1) 993 2) 990	1) 90.2 2) 90.0	1) 6.1 2) 8.9
2 ♀	6/ 4/46	8.9	1) 32.2 2) 31.5 3) 29.0	1) 6.16 2) 6.16 3) 5.66	1) 96 2) 88 3) 92	1) 604 2) 652	1) 67.8 2) 73.2	+8.00	1) 909 2) 967	1) 102 2) 109	1) 11.1 2) 9.0
3 ♂	4/14/46	9.5	1) 42.5 2) 40.5 3) 39.6	1) 6.06 2) 5.71 3) 5.50	1) 68 2) 64 3) 64	1) 785 2) 810	1) 82.5 2) 85.2	+3.3	1) 1376 2) 1380	1) 145 2) 145	1) 13.3 2) 5.2
4 ♀	2/ 4/46	9.6	1) 40.4 2) 42.0 3) 40.4	1) 6.62 2) 6.26 3) 5.61	1) 108 2) 116 3) 108	1) 572 2) 595	1) 59.5 2) 62.0	+4.20	1) 1058 2) 1011	1) 110 2) 105	1) 9.9 2) 12.5
5 ♀	4/10/46	6.7	1) 34.7 2) 32.6 3) 32.5	1) 6.21 2) 5.86 3) 5.45	1) 92 2) 96 3) 88	1) 527 2) 566	1) 78.6 2) 84.5	+7.50	1) 824 2) 866	1) 123 2) 129	1) 11.2 2) 6.8
6 ♀	5/ 2/46	7.0	1) 42.1 2) 42.7 3) 41.7	1) 7.77 2) 7.77 3) 7.67	1) 72 2) 72 3) 76	1) 471 2) 457	1) 67.4 2) 65.5	−2.8	1) 855 2) 792	1) 122 2) 113	1) 8.3 2) 6.0
7 ♀	4/29/46	13.2	1) 39.8 2) 38.6 3) 38.0	1) 6.62 2) 6.52 3) 6.42	1) 55 2) 60 3) 64	1) 602 2) 626	1) 45.6 2) 47.5	+4.2	1) 1031 2) 1044	1) 78.4 2) 79.0	1) 6.4 2) 4.1
8 ♀	6/10/46	9.0	1) 41.2 2) 40.8 3) 40.1	1) 6.36 2) 6.11 3) 5.91	1) 112 2) 100 3) 92	1) 640 2) 652	1) 71.0 2) 72.4	+2.0	1) 1107 2) 1113	1) 123 2) 124	1) 13.8 2) 10.2
9 ♀	3/25/46	7.7	1) 44.9 2) 43.1 3) 42.5	1) 6.62 2) 6.36 3) 6.01	1) 120 2) 112 3) 112	1) 452 2) 498	1) 59.7 2) 64.6	+8.2	1) 834 2) 900	1) 108 2) 117	1) 15.0 2) 7.0
10 ♀	6/ 4/46	8	1) 44.2 2) 44.6 3) 42.4	1) 6.47 2) 6.16 3) 6.01	1) 96 2) 96 3) 88	1) 354 2) 453	1) 44.4 2) 56.6	+27.4	1) 660 2) 827	1) 82.5 2) 103	1) 21.6 2) 4.5
11 ♀	3/27/46	8.4	1) 34.6 2) 33.1 3) 32.9	1) 7.62 2) 7.42 3) 7.32	1) 60 2) 56 3) 56	1) 720 2) 725	1) 85.5 2) 86.3	+ .94	1) 1119 2) 1097	2) 134 2) 130	1) 11.9 2) 14.4

(Continued on following page)

TABLE II (continued).

Dog No. and sex	Date	Wt, kg	Hct × .96, %	Plasma protein, % (refract)	Heart rate/min.	Plasma vol, ml	Plasma vol, ml/kg	Dev. of 2nd plasma vol from 1st plasma vol, %	Blood vol, ml	Blood vol, ml/kg	Disappearance rate, %/hr
12 ♀	6/10/46	9.2	1) 46.5 2) 43.5 3) 42.0	1) 7.17 2) 6.87 3) 6.47	1) 140 2) 136 3) 144	1) 464 2) 524	1) 50.5 2) 57.0	+12.8	1) 920 2) 940	1) 100 2) 102	1) 17.9 2) 6.4
Mean		9.01	1) 40.6	1) 6.64		1) 562	1) 63.6 ±14.2 s.d.	+ 6.4 ±7.8 s.d.	1) 973.8	1) 109.8 ±20.4 s.d.	1) 12.2
			2) 39.6	2) 6.42		2) 593	2) 67.1 ±13.4 s.d.		2) 993.9	2) 112.2 ±18.3 s.d.	2) 7.9
			3) 38.5	3) 6.12							

* See footnote Table I.

obtains. Loss rates can obviously be influenced by regional distribution of blood and rates of flow as well as by "gobbling" mechanisms; and on distribution and flow we have no more information in our experiments than did Cruickshank and Whitfield. Cruickshank and Whitfield also show one test (how many others were done is not reported) with small and large injections of dye purporting to indicate that "the amount of dye taken up by the absorbing system is practically independent of the concentration of dye in plasma" (1). This question was investigated extensively in dogs by Allen and Gregersen(3) who found satisfactory agreement in volumes determined in succession first with small and then with excessively large amounts of T-1824 giving plasma concentrations in excess of 1 mg/ml.

While no one denies that the reticulo-endothelial system and other tissues take up dye, the crux of the matter is whether or not this absorption is sufficient during the "mixing period" to invalidate the extrapolation method for determining the theoretical value C_0 used in calculating plasma volume. The strongest kind of circumstantial evidence supports extrapolation as a valid method of correcting for the loss of dye (and other test substances) during mixing. Briefly this evidence in part consists of a) the experiments of Allen and Gregersen(3) and b) the repeated demonstration that in those species in which T-1824 is firmly bound to albumin this dye has the same volume distribution as various other test substances including bovine albumin, bovine globulin, the polysaccharide S III, hemoglobin, I^{131} tagged albumin(4) and dextran (5,6,7).

The decreases in hematocrit values and plasma protein concentrations shown in Tables I and II are of interest here chiefly because they accord both in direction and magnitude with the changes in plasma volume measured by successive injections of T-1824.

Conclusion. In animal species in which T-1824 is firmly bound to the plasma proteins, absorption of dye by the reticulo-endothelial system and other tissues during the "mixing period" is not a significant source of error in measurement of plasma volume with this dye. With T-1824, as well as with other suitable test substances, back-extrapolation of the time concentration curve on a semi-log plot provides satisfactory correction for loss during the mixing period.

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Received July 6, 1960. P.S.E.B.M., 1960, v104.

F_{cells} Values in the Normal and Splenectomized Cat: Relation of F_{cells} to Body Size. (25968)

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The purpose of the present investigation was to carry out simultaneous measurements of plasma and cell volume in normal and splenectomized cats with T-1824 and Cr^{51} to determine the F_{cells}† value in this species and to study the effect of filling and emptying of the spleen on the F_{cells} factor.

Materials and methods. Fourteen healthy male cats were selected for the studies which covered the period from Sept., 1958 to May, 1959. All cats were given anti-feline distemper serum and acromycin and kept for one month under uniform laboratory conditions before making any observations. Nine cats were then splenectomized and allowed to recuperate for 4 to 5 weeks before attempting measurements on blood volume. With one exception, 2 determinations were done on each cat and these were made not less than 10 days apart to avoid the effect of previous blood sampling on the results. It should be noted that the procedures were designed to limit blood sampling to 5 to 10 ml for a complete determination of both red cell volume and plasma volume.

Since the spectral absorption curve of T-1824 is different in the plasma of various animals(1), the dye was standardized in cat plasma. Values for optical density of a 1:500 dilution of 0.5% T-1824 (Warner, lot No. 046033) in plasma were obtained on a Beckman B spectrophotometer using 10 mm absorption cells. The peak of spectral absorption on our instrument was at 630 mu. At this wave length average optical density was 0.80 (s.d. ± 0.035). Hematocrit determinations were done by centrifugation at 3000 rpm (radius 19 cm) for 30 minutes. The trapping factor was determined by the method described by Wang(2). In 12 experiments, average value for the trapping factor was found to be 0.96 with a standard deviation of ± 0.01.

Utilizing the methods described below, a series of beaker experiments were performed on known amounts of cat blood. The overall error in these *in vitro* experiments was well under ± 2%.

On the day before each experiment, a 2 ml sample of blood was withdrawn from the cat and incubated at 37°C for 45 minutes with approximately 20 μc of Abbott's rachromate. The cells were then separated from the plasma by centrifugation and carefully washed 2 or 3 times with cold isotonic saline to remove unbound Cr^{51} and stored in the icebox for subsequent use.

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† $F_{cells} = \frac{\text{overall cell }\%}{\text{venous cell }\%}$.

All animals were given water *ad libitum* but no food for at least 12 hours before experiment to avoid the interference of lipemia in the optical density readings of T-1824. For experiments under anesthesia, 30 mg of Nembutal per kilo of body weight were given intraperitoneally. In experiments without anesthesia the cat was placed in a supine position on a cat board and tied down as lightly as possible to prevent obstruction of the circulation as described by Hamlin and Gregersen (3). After the cats became accustomed to this routine. blood sampling generally proceeded smoothly.

The amount of T-1824 injected was measured by weighing a tuberculin syringe before and after filling with about 0.20 ml of dye. The radioactive cells which had been prepared on the preceding day were washed once again and resuspended in cold isotonic saline. Just prior to injection a calibrated 2 ml syringe was used to withdraw an aliquot sample of the labeled cells. At the same time, 3 standards were prepared by placing 1 ml aliquot samples of the cell suspension in each of 3 calibrated 25 ml volumetric flasks which were then filled to the mark with distilled water to produce hemolysis. One ml aliquots of the standards were later counted in an Atomic Associates multiscaler counter, model 1071. From the average result, number of counts per ml of cell suspension and total number of counts injected were calculated.

Using a heparinized syringe, 1.5 ml of blood was withdrawn from the femoral vein to serve as a "blank" for the cell and plasma volume determinations. The needle was left in the vein and the blank sample promptly transferred to a 4 ml hematocrit tube. The tagged cells were injected first, the dye injection following immediately thereafter and the times noted. The syringe used for radioactive cells was rinsed in 20 ml of distilled water and a 1 ml aliquot sample was counted to calculate the residual activity in the syringe. This value was subtracted from total number of counts originally in the syringe to determine the actual number of counts injected into the cat. Likewise, the dye syringe (and the needle) were withdrawn and rinsed in 10 ml of saline. The rinse was then centrifuged and the clear, blue-tinged supernatant fluid was read against saline in the Beckman B spectrophotometer at a wave length of 620 mμ to calculate amount of residual dye in the syringe. This was subtracted from the original weight of dye in the syringe to determine actual amount of dye injected.

Following the injections of labeled cells and dye, five 1.5 ml samples were drawn from a femoral vein with heparinized syringes at about 10 minute intervals and transferred to 4 ml hematocrit tubes. These were centrifuged and hematocrit values recorded and corrected, using the trapping factor of 0.96 to obtain the cell percentage for each sample. The plasma was pipetted off and centrifuged to insure a clear solution for the optical density readings which were made in micro cells against the "blank" plasma. Plasma protein concentrations in all samples were calculated from refractive index readings on the Abbe refractometer. The time concentration curve of the dye was plotted using appropriate corrections for fluid shifts as revealed by the plasma protein readings and plasma volume calculated by back extrapolation in the usual manner(4).

A very simple procedure was devised for preparation of cell samples for counting. The plasma that had been removed from the hematocrit tube was *exactly* replaced by a 1% saponin solution using a fine pipette. One ml from each sample of the hemolyzed cells was then counted and from this and from the cell percentage the counts per ml of cells can be accurately calculated. Total cell volume was determined by dividing this value into total number of counts injected.

From the determined values of plasma volume, cell volume and venous cell percentage, the following calculations were made:

$$\text{Total blood volume} = \text{Cell volume} + \text{plasma volume}$$

$$\text{Overall cell \%} = \frac{\text{Total cell volume}}{\text{Total blood volume}}$$

$$F_{cells} = \frac{\text{Overall cell \%}}{\text{Venous cell \%}}$$

Results. Table I shows the results of 2 plasma volume determinations on 9 splenec-

TABLE I. Plasma Volumes on Splenectomized Cats under Nembutal.

Cat No.	N_1*	Body wt (kg)	PV (ml)
1	2	2.93	37.8
2	2	3.23	40.5
3	2	3.25	34.8
4	2	3.38	42.1
6	2	4.19	37.7
7	2	3.30	38.4
8	2	3.06	42.4
9	2	2.84	43.9
10	2	3.66	41.4
Mean of 9 cats and S.D.			39.9 ± 2.8

* N_1, No. of observations on each animal.

tomized cats anesthetized with Nembutal. The average plasma volume per kilo body weight is 39.9 (± 3.0). In Table II, we have tabulated the results of simultaneous measurements with T-1824 and Cr^{51} on 7 of the same cats when unanesthetized. (Two of the animals had died before these tests could be completed.) In this series mean plasma volume is 36.65 ml/kg, cell volume (RCV) 11.8 ml/kg, total blood volume 50.3 ml/kg. The calculated F_{cells} values fall within a narrow range from 0.76 to 0.80 with a mean of 0.78 ± .01. It should be noted that the average disappearance rates of T-1824 in anesthetized and unanesthetized animals were 14.8 and 15.4% per hour respectively.

The upper part of Table III shows the results of simultaneous cell and plasma volume determinations from 5 normal cats (spleen intact) under Nembutal anesthesia before and after injections of adrenaline. The lower part of Table III gives results of the same tests on 3 splenectomized cats, also under Nembutal. In the former group before injection of adrenaline, F_{cells} values ranged from 0.88 to 1.06. In each instance the F_{cells} value falls markedly after adrenaline is given, while venous cell percentage is seen to rise. In the 3 anesthetized splenectomized cats, F_{cells} value ranged from 0.79 to 0.83 and these values hardly change after injection of adrenaline, nor is there any marked change in venous cell percentage.

The contrast in the effects of adrenaline on F_{cells} value and venous cell percentage in the normal and splenectomized cats is shown graphically by the typical experiments plotted in Fig. 1 and 2.

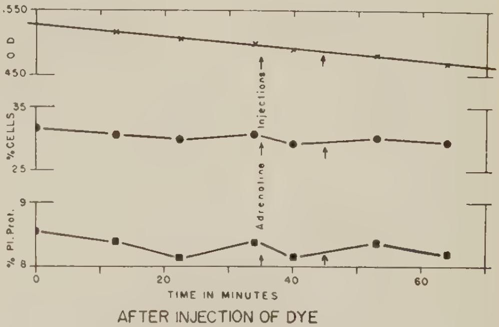

FIG. 1. Results from a typical experiment on an unanesthetized splenectomized cat showing the characteristic time-concentration curve of T-1824, and the minor variations in both venous cell percentage and plasma protein concentration. Cr^{51} counts not included in the diagram were constant within ±1.0% throughout. Note that the 2 adrenaline inj. (indicated by the arrows) had little if any effect on the variables shown.

Discussion. When using either the plasma volume-hematocrit or the cell volume-hematocrit method for "estimating" total blood volume, the F_{cells} factor $\left(\frac{\text{overall cell \%}}{\text{venous cell \%}}\right)$ must be known and included in the calculation

TABLE II. Simultaneous Determinations of Plasma Volume and Cell Volume on Splenectomized, Unanesthetized Cats.

Cat No.	N_1*	Body wt, kg	PV	RCV	TBV	Venous cell	Overall cell	F_{cells}
				ml/kg			%	
2	2	3.10	41.5	11.0	52.4	27.2	20.8	.77
3	2	3.20	32.5	11.5	44.7	32.7	25.8	.79
6	1	3.60	33.7	11.8	46.2	33.1	25.5	.77
7	2	3.09	33.6	11.5	50.6	29.8	22.8	.76
8	2	2.70	35.6	12.5	48.5	32.1	25.7	.80
9	2	2.78	43.8	12.6	57.1	28.4	22.0	.77
10	2	3.80	35.9	11.7	52.8	28.5	22.2	.78
Mean			36.7	11.8	50.3	30.3	23.5	.78 ± .01†

* N_1, No. of observations on each cat.

† Stand. dev.

TABLE III. The Effect of Adrenaline* on F_{cells} Value and Venous Cell Percentage.

Cat No.	Date	Body wt, kg	PV ml/kg	RCV ml/kg	TBV ml/kg	Venous cell %	Overall cell %	F_{cells}
				Normal, anesthetized† cats				
5	2/19/59	2.79	34.6	17.7	52.3	35.5 *45.7*	33.6 *33.6*	.95 *.74**
11	2/18/59	3.48	44.9	16.8	61.7	25.4 *36.7*	26.8 *26.8*	1.06 *.73*
13	3/ 5/59	3.23	36.9	13.7	50.6	30.7 *40.3*	26.9 *26.9*	.88 *.67*
15	3/ 3/59	3.33	52.0	13.7	65.7	20.7 *33.9*	20.8 *20.8*	1.00 *.61*
16	3/25/59	3.57	35.1	12.2	47.3	27.0 *38.5*	25.7 *25.7*	.95 *.67*
				Splenectomized, anesthetized† cats				
3	1/16/59	3.3	33.6	12.7	46.3	31.1 *32.2*	25.8 *25.8*	.83 *.80*
3	2/10/59	3.48	33.0	12.8	45.8	33.5 *31.9*	26.4 *26.4*	.79 *.83*
2	2/ 4/59	3.3	39.6	13.7	53.3	31.3 *32.3*	25.6 *25.6*	.82 *.79*

* Values obtained after injections of adrenaline are shown in italics. For typical spacing of injections, see Fig. 1 and 2.

† Nembutal, 30 mg/kg intraper.

to arrive at a true estimate for total blood volume(4). The present experiments show that in the splenectomized cat this F_{cells} factor is 0.78 (s.d. ± .01).

From the experimental results given in Table III it is obvious that in cats with spleen intact, as in normal dogs(5), "estimates" of blood volume from measurement of either cell volume or plasma volume alone are unreliable since the F_{cells} factor is highly variable depending on the state of the spleen.

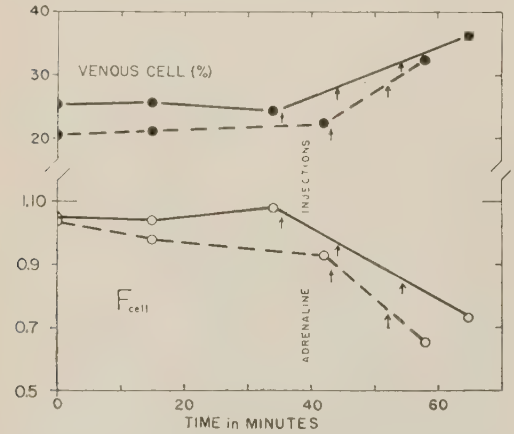

FIG. 2. Graphic presentation of effects of adrenaline inj. (arrows) on venous cell percentage and F_{cells} value in cat #11 (solid line) and cat #15 (broken line) from Table III.

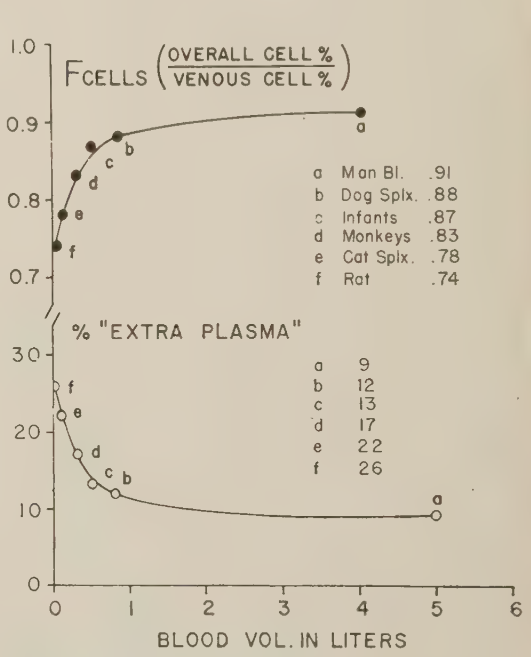

FIG. 3. Showing that the observed differences in F_{cells} values (upper curve) and "extra plasma" (lower curve) in various species are related to body size. Revised from figure published previously (Gregersen, M. I., *Chinese Med. J.*, 1959, v6, 171).

The mean F_{cells} value of 0.78 in splenectomized cats agrees with other evidence that species differences in the F_{cells} value may be fundamentally related to body size. This is shown in Fig. 3 originally constructed from F_{cells} values from the literature tabulated by Gregersen and Rawson(4) and here revised to include the final value obtained in the splenectomized cat. It is apparent from Fig. 3 that the smaller the animal, and hence the smaller the F_{cells} value, the greater is the deviation in uncorrected "estimates" of total blood volume from the "true" or "measured" blood volume. This fact largely explains the differences between the "measured" total blood volumes on cats reported here and uncorrected "estimates" reported by Neidle and Gregersen(6) because the mean of the measured plasma volume (in ml/kg) in their study is in close agreement with ours, the two values being 41.0 ml/kg and 39.9 ml/kg respectively.

Summary. The F_{cells} factor in the splenectomized cat is 0.78 (s.d. ± .01), but with spleen intact the F_{cells} factor is highly variable as shown by the striking effects of adrenaline on the F_{cells} value in the normal anesthetized cat. Evidence is presented indicating that species differences in the F_{cells} value may be fundamentally related to body size.

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Blood Volume in Sympathectomized-Splenectomized Dogs.* (25969)

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When both plasma and cell volumes are determined simultaneously, one obtains a true measure of total blood volume and can calculate the overall cell percentage(1). The ratio of overall cell percentage/large vessel cell percentage, or F_{cells} factor, is quite constant in normal splenectomized dogs and averages 0.87 or 0.88(2-4). The most reasonable explanation for an F_{cells} factor lower than unity is that the cell percentage of the blood in small vessels is lower than that in the large arteries and veins(1). Thus, the F_{cells} value would depend upon the relative volume, as well as relative cell percentage, of blood in the 2 compartments, namely the small vessels and the large vessels. Since peripheral circulation is under the control of sympathetic nervous system, it is interesting to see if the removal of sympathetic activity would alter distribution of cells and plasma, which is reflected in the F_{cells} value. A few measurements of cell and plasma volumes in sympathectomized-splenectomized dogs were briefly reported in a previous paper dealing with the circulatory adjustment to hemorrhage(5). In the present study, 33 measurements of blood volume were made on 20 sympathectomized-splenectomized dogs. The F_{cells} factor, as well as cell volume, plasma volume, arterial cell percentage, and plasma protein concentration, are reported and compared with those in splenectomized dogs with sympathetics intact.

Material and methods. Nine male and 11 female mongrel dogs were used. Total thoracolumbar sympathectomy and splenectomy were performed as described earlier(5), at least 4 weeks before the experiments. Blood volume was measured without general anesthesia. Preparation of the animal, measurement of cell volume with Cr^{51} and plasma volume with T-1824, and determination of hematocrit and plasma protein concentration were carried out in the manner reported previously(4).

Results. The results are listed in Table I. Arterial cell percentage is calculated from the

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* Investigation supported in part by grant from China International Fdn.

TABLE I. Blood Volume in Sympathectomized-Splenectomized Dogs.

Dog No.	Date of last op.	Exp. date	B.W. (kg)	Art. cell %	P.P. (g %)	PV	CV	BV	F_{cells} factor	PV	CV	BV
							(ml)				(ml/kg)	
					A. Male dogs							
1	5/17/56	6/19/56	9.8	42.5	6.62	531	298	829	.838	54.2	30.4	84.6
		9/22/56	10.5	41.4	6.01	535	342	877	.915	51.0	32.6	83.5
		11/16/56	10.6	40.9	6.21	575	345	920	.917	54.2	32.5	86.8
2	7/10/56	8/ 7/56	7.6	46.6	7.32	428	277	707	.845	56.3	36.6	92.9
		9/15/56	8.2	36.0	6.47	522	245	767	.862	63.7	29.9	93.5
		12/ 3/56	8.6	38.7	6.87	538	284	822	.871	62.6	33.0	95.6
4	10/ 8/56	11/16/56	9.9	43.6	6.21	484	296	780	.871	48.9	29.9	78.8
		1/ 3/57	10.7	45.8	6.47	508	352	860	.893	47.5	32.9	80.4
7	6/11/58	7/ 9/58	11.6	45.4	6.26	559	373	932	.881	48.2	32.2	80.3
		8/ 4/58	11.7	43.0	6.31	647	391	1038	.877	55.3	33.4	88.7
8	6/17/58	7/18/58	9.7	42.1	5.86	480	292	772	.898	49.5	30.1	79.7
		3/24/59	10.0	43.8	5.66	447	277*	724	—	44.7	27.7	72.4
9	6/30/58	7/31/58	9.6	37.5	6.56	583	278	861	.861	62.3	30.6	93.0
		8/25/58	9.7	40.0	6.42	604	297	901	.825	60.8	29.0	89.8
12	7/29/58	8/27/58	10.7	41.2	6.62	682	378	1060	.867	63.7	35.3	99.1
		12/29/59	12.9	49.8	6.47	607†	469	1076	—	47.2	36.3	83.5
13	2/10/59	4/22/59	10.0	45.7	6.31	545	341	886	.848	54.5	34.1	88.6
16	3/12/59	5/29/59	10.8	41.6	6.42	673	357	1030	.832	62.3	33.0	95.4
	Mean		10.1	42.5	6.39				.869	54.8	32.2	87.0
	Stand. dev.		1.26	3.41	.37				.028	6.44	2.47	7.15
					B. Female dogs							
3	7/26/56	8/23/56	8.1	36.8	5.61	418	196	614	.870	51.6	24.3	75.9
		11/ 4/56	7.9	43.9	7.02	423	261	689	.863	54.2	33.0	87.2
5	11/16/56	2/ 5/57	9.1	34.4	6.31	582	257	839	.892	64.0	28.2	92.2
6	1/22/57	2/24/57	8.6	41.0	6.26	482	277	759	.890	56.0	32.2	88.2
10	7/17/58	8/13/58	10.7	41.4	5.96	547	302	849	.860	51.1	28.2	79.3
		8/29/58	10.9	42.7	5.81	636	360	996	.846	58.3	33.0	91.3
		4/17/59	11.5	41.7	6.36	556	320	876	.875	48.3	27.8	76.1
11	7/15/58	8/15/58	7.2	45.5	5.35	377	223	600	.819	52.3	31.0	83.3
		11/10/58	8.5	47.4	5.76	386	260	646	.848	45.5	30.6	76.0
14	2/17/59	4/24/59	7.5	45.4	6.16	391	253	644	.866	52.1	33.8	85.9
15	3/ 3/59	5/15/59	11.5	41.3	6.21	608	328	936	.850	52.9	28.5	81.4
17	6/25/59	8/ 5/59	7.6	39.4	5.15	363	179	542	.838	47.7	23.6	71.3
18	6/29/59	8/13/59	9.4	47.1	5.30	538	375	914	.870	57.2	39.9	97.1
19	7/13/59	9/ 3/59	9.3	44.7	6.26	432†	276	708	—	46.5	29.7	76.2
20	7/21/59	11/19/59	8.1	41.9	6.26	407†	234	641	—	50.2	28.9	79.1
	Mean		9.1	42.3	5.99				.861	52.5	30.2	82.7
	Stand. dev.		1.46	3.61	.49				.020	4.91	4.00	7.40

* CV calculated from PV, art. cell %, and an assumed F_{cells} factor of .87(4).
† PV " " CV, *Idem*.

ratio of height of red cell column plus buffy coat to total sample height in the hematocrit tube, with correction for the plasma trapping factor 0.96(6). Plasma protein concentration is computed from the refractive index(7). Cell volume (CV) is calculated from the red cell volume (RCV) with the formula:

$$CV = RCV \times \frac{\text{arterial cell \%}}{\text{arterial red cell \%}}.$$

Blood volume (BV) is obtained by summing plasma volume and cell volume. The F_{cells} factor is computed from the formula:

$$F_{cells} = \frac{CV/BV}{\text{arterial cell \%}} \times 100.$$

Discussion. 1. Comparison of male and female dogs. Average PV/kg, CV/kg, and BV/kg are all higher in the male animals (Table I). This is what one would expect from the findings in man(8). These sex differences in the present study, however, are not statistically significant ($P > 0.05$ with t test for all 3 volumes). The arterial cell percentage is al-

TABLE II. Analysis of Variance for Male Sympathectomized-Splenectomized Dogs.

Nature of measurement	Source of variation	Sum of squares	Degrees of freedom	Mean square	F-ratio	s	s_A	$\sqrt{s^2 + s_A^2}$
CV, ml/kg	Among dogs	68.67	8	8.58	2.18			
	Composite error	35.46	9	3.94				
	Total	104.13	17					
	Stand. dev.					1.98	1.53	2.51
PV, "	Among dogs	491.33	8	61.42	2.59			
	Composite error	213.69	9	23.74				
	Total	705.02	17					
	Stand. dev.					4.88	4.39	6.55
BV, "	Among dogs	662.42	8	82.80	3.60*			
	Composite error	207.28	9	23.03				
	Total	869.70	17					
	Stand. dev.					4.80	5.50	7.30
F_{cells} factor	Among dogs	.00615	8	.000781	1.03			
	Composite error	.00530	7	.000758				
	Total	.01145	15					
	Stand. dev.					.0275	.00364	.0278

* Significant at .05 level.

most identical in the 2 groups, whereas plasma protein concentration is higher ($P < 0.02$) in the male. The F_{cells} factor, 0.869 for the male and 0.861 for the female, is almost the same in the 2 groups ($P > 0.3$).

2. Analysis of variance(9). Since repeated measurements were made on several male dogs, one can analyze and study the components of variance in this group (Table II). Two possible components of variation can be isolated from these experiments. One component, which is estimated as s^2 (mean square for 'composite error' in Table II), is that resulting from repeat observations made on the same dog. It may include measurement error, dietary effects, seasonal and age changes, etc. Another component, estimated as s_A^2, is that resulting from observations made on different dogs and reflects biological variability. If there are true differences among these male dogs as far as can be detected here, then the mean square for 'among dogs' should contain the second component of variance estimated by s_A^2, in addition to the first component estimated by s^2 (mean square for 'composite error'). The F-ratio (mean square for 'among dogs'/mean square for 'composite error') thus tests the extent to which differences among dogs exceed the error in the same dog. The magnitude of the F-ratio for various measurements is in the order: BV/kg, PV/kg, CV/kg, and F_{cells} factor (Table II). From the F-ratio and a comparison of s and s_A (Table II), it would appear that the individual differences for F_{cells} factor are no greater than would be expected from chance alone. For blood volume, on the other hand, these male sympathectomized-splenectomized dogs appear to exhibit true biological differences. These findings are similar to those in the rhesus monkey(10).

3. Comparison of sympathectomized with non-sympathectomized dogs. In Table III, the results for the male sympathectomized-splenectomized dogs in the present study are compared with those of 22 male splenectomized dogs subjected to the same experimental procedures(4). The sympathectomized dogs have higher averages for PV/kg, CV/kg, and BV/kg. The differences are significant at the 5% level for cell volume and blood volume, but not for plasma volume. In an earlier report on a smaller number of animals, sympathectomized-splenectomized dogs(5) were also found to have a higher blood volume per unit body weight (85.9 ml/kg) than splenectomized dogs (81.4 ml/kg). The difference in blood volume in that study, however, was entirely attributable to plasma volume (sympathectomized - splenectomized, 56.1 ml/kg; splenectomized, 50.8 ml/kg). The discrepancy between the 2 series in this aspect is difficult to explain. For one thing, the comparison in the earlier report(5) was made without regard to sex. Difference in nutritional status, seasonal variations and age may also be contributory factors. Comparison of data on the same animal before and after sympathectomy would probably be more conclusive than the

TABLE III. Comparison of Sympathectomized-Splenectomized and Splenectomized Dogs.

		B.W. (kg)	Art. cell %	P.P. (g %)	PV	CV (ml/kg)	BV	F_{cells} factor
A. Splenectomized*	Mean	10.6	41.5	6.18	52.2	30.2	82.4	.88
	S.D.		2.72	.30	6.02	3.12	7.48	.020
B. Sympathectomized-splenectomized	Mean	10.1	42.5	6.39	54.8	32.2	87.0	.87
	S.D.		3.41	.37	6.44	2.47	7.15	.028
Mean B – Mean A			+1.0	+.18	+2.6	+2.0	+4.6	–.01
t			1.03	1.69	1.32	2.21	1.97	1.10
P			.3	.1	.2	.04	.05	.3

* Data taken from 22 male splenectomized dogs reported by Rawson, Chien, Peng and Dellenback(4).

comparison of population means.

Arterial cell percentage, plasma protein concentration and F_{cells} factor for the 2 groups of animals show no significant difference ($P > 0.05$, Table III). An unchanged F_{cells} value after sympathectomy would indicate that the removal of sympathetic system does not cause marked changes in distribution of cells and plasma in the large and small vessels. Although sympathectomy causes some dilatation of blood vessels and tends to increase the volume of blood in the small vessels, the result on F_{cells} factor suggests that this increase is either not very large or is accompanied by a concomitant decrease in cell percentage in these vessels.

Summary. With the use of Cr^{51} and T-1824, cell and plasma volumes are measured in sympathectomized-splenectomized dogs. Average cell volume, plasma volume, and blood volume are 32.2 ml/kg, 54.8 ml/kg, and 87.0 ml/kg respectively for the male dogs; and 30.2 ml/kg, 52.5 ml/kg, and 82.7 ml/kg respectively for the females. These sex differences are not statistically significant. A comparison between sympathectomized-splenectomized dogs and splenectomized dogs shows higher cell and blood volumes (significant at the 5% level) for the former group. Analysis of variance demonstrates significant individual differences in blood volume among the male sympathectomized-splenectomized dogs. The F_{cells} factor shows neither significant individual difference nor sex difference, and is unchanged by sympathectomy.

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Received July 6, 1960. P.S.E.B.M., 1960, v104.

F_{cells} Value in Sympathectomized-Splenectomized Dogs after Hemorrhage.* (25970)

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The F_{cells} factor (overall cell percentage/venous cell percentage) is quite constant in splenectomized dogs, and is not apparently changed by variations in hematocrit level, transfusion or hemorrhage(1-3). It is well known that cardiac stimulation and vasoconstriction due to sympathetic activity constitute important circulatory adjustments to hemorrhage(4,5). Such compensatory mechanisms may conceivably adjust the distribution of cells and plasma in the circulation and contribute to the constancy of F_{cells} value after hemorrhage. To test this possibility, the F_{cells} factor in sympathectomized-splenectomized dogs is determined after hemorrhage and compared with the prehemorrhage value, which has been shown to be the same as that in splenectomized dogs(6).

Materials and methods. Twenty-five experiments were done on 19 unanesthetized mongrel dogs, sympathectomized and splenectomized(5) at least 4 weeks before experiments. Preparation of the animal, measurement of cell and plasma volumes, determination of hematocrit and plasma protein concentration, and removal of blood were performed as described elsewhere(3). The control blood volume of these experiments is given in the accompanying paper(6). One to 2 hours after removal of 146 to 362 ml of blood (19 to 43% of the control blood volume), posthemorrhage blood volume was measured.

Results. In Table I, results are tabulated in the descending order of percent of control blood volume remaining when posthemorrhage measurements were made. Posthemorrhage cell volume was either measured directly with Cr^{51} (CV in Table I), or calculated as the difference between control cell volume and volume of cells removed (CV′). In 6 dogs, both CV and CV′ were determined, and they show very good agreement (Table I). In 19 of these 25 experiments, posthemorrhage F_{cells} value was within the control range of 0.82 to 0.92(6). The other 6 dogs had values lower than 0.80 after hemorrhage, the lowest value being 0.725 (dog #15). The relation between F_{cells} factor and % of initial blood volume remaining is shown in Fig. 1. The 6 low values were obtained in experiments where blood volume was reduced to 73-77% of the control.

Discussion. 1. F_{cells} factor after hemorrhage. Although the F_{cells} value is unchanged in splenectomized dogs after hemorrhage(1,3) or sympathectomy(6), it decreases in some sympathectomized-splenectomized dogs after hemorrhage. Therefore, the sympathetic activity after hemorrhage probably aids in redistribution of cells and plasma between large and small vessels such that a relationship similar to the prehemorrhage one can exist. That the sympathetic activity is not the only factor involved in this redistribution is evidenced by the fact that most sympathectomized-splenectomized dogs still have F_{cells} values after hemorrhage comparable to the control. Zweifach (7) has shown that the compensatory behavior to hemorrhage of the small vessels in

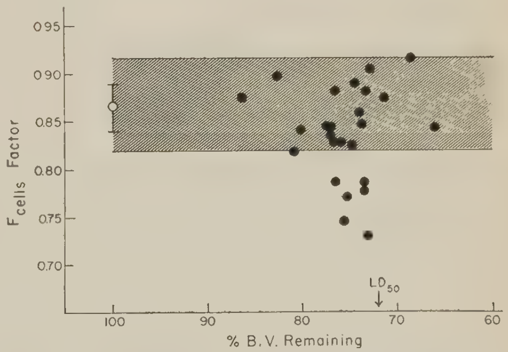

FIG. 1. Relationship between posthemorrhage F_{cells} factor and percent of control blood volume remaining. Open circle and vertical bars denote mean and stand. dev. of control F_{cells} factor. The two horizontal lines across the graph represent range of control F_{cells} factor. Note the 6 low F_{cells} values obtained in a range of 73-77% blood volume remaining. LD_{50} for sympathectomized-splenectomized dogs after hemorrhage is found with 72% of blood volume remaining(8), as indicated by the arrow.

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* Investigation supported in part by grant from China International Fdn.

TABLE I. F_{cells} Factor in Sympathectomized-Splenectomized Dogs after Hemorrhage.

			Control				Posthemorrhage						
Dog No. and sex	Date of exp.	Body wt (kg)	Art. cell %	BV (ml)	F_{cells}	Hemor. vol (ml)	Art. cell %	PV	CV	CV′	BV	BV %	F_{cells} factor
								(ml)					
8 ♂	7/18/58	9.7	42.1	772	.898	146	41.3	426	—	241	667	86.4	.875
6 ♀	3/ 6/57	8.5	42.1	793*	.890†	155	40.0	420	235	—	655	82.6	.897
14 ♀	4/24/59	7.5	45.4	644	.866	187	42.4	340	184	177	521	80.8	.819
2 ♂	12/ 9/56	9.1	37.3	858*	.859†	209	34.1	491	198	—	690	80.1	.842
13 ♂	4/22/59	10.0	45.7	886	.848	255	41.2	441	234	234	675	77.4	.844
4 ♂	1/13/57	11.3	44.2	811*	.882†	247	39.3	419	208	—	627	77.3	.844
10 ♀	4/17/59	11.5	41.7	876	.875	267	37.0	467	208	210	676	77.1	.836
19 ♀	9/ 3/59	9.3	44.7	708	—	211	41.0	357	—	186	543	76.7	.828
1 ♂	11/24/56	10.0	41.4	880*	.890†	236	38.2	446	227	—	673	76.5	.882
20 ♀	11/19/59	8.1	41.9	641	—	193	39.4	338	149	155	490	76.5	.787
3 ♀	2/ 6/57	9.0	38.3	757*	.866†	252	33.4	416	159	—	575	76.0	.828
11 ♀	8/15/58	7.2	45.5	600	.819	201	40.2	318	—	136	454	75.7	.745
8 ♂	3/24/59	10.0	43.8	724	—	215	41.4	372	174	—	546	75.4	.771
9 ♂	8/25/58	9.7	40.0	901	.825	255	36.5	472	—	203	675	74.9	.825
5 ♀	1/ 8/57	9.3	35.7	782*	.892†	196	34.8	402	181	—	583	74.5	.891
12 ♂	8/27/58	10.7	41.2	1060	.867	356	36.1	542	—	244	786	74.1	.859
9 ♂	7/31/58	9.6	37.5	861	.861	241	34.9	448	—	188	636	73.9	.848
17 ♀	8/ 5/59	7.6	39.4	542	.838	180	36.4	286	—	113	399	73.6	.777
10 ♀	8/13/58	10.7	41.4	849	.860	328	35.5	450	—	174	624	73.5	.786
18 ♀	8/13/59	9.4	47.1	914	.870	285	42.5	420	—	251	671	73.4	.880
15 ♀	5/15/59	11.5	41.3	936	.850	379	36.0	505	174	182	683	73.0	.725
7 ♂	8/ 4/58	11.7	43.0	1038	.877	340	36.7	505	—	251	756	72.8	.905
12 ♂	12/29/59	12.9	49.8	1076	—	362	44.5	469	299	298	768	71.4	.874
10 ♀	8/29/58	10.9	42.7	996	.846	339	36.5	454	—	229	683	68.6	.918
11 ♀	11/10/58	8.5	47.4	646	.848	277	39.5	284	—	142	426	66.2	.843

* Control BV calculated from the equation $BV_1 = \frac{\text{Posthemor. CV} + \text{CV out}}{.87 \times \text{control art. cell \%}} \times 100$.

† Control F_{cells} value obtained at a different date.

the mesentery is a composite of the activity of renal, adrenal, and sympathetic homeostatic mechanisms superimposed on the intrinsic tone of the blood vessels themselves.

The critical level (LD_{50}) for sympathectomized-splenectomized dogs to survive hemorrhage is when blood volume is reduced to 72% of the control(8). This is close to the range of remaining blood volume where the low F_{cells} values are found. The decrease in F_{cells} value may be due to a relative increase in volume of blood in the small vessels and/or a decrease in cell percentage there. It cannot be decided whether one or both of these factors are operating. But pooling of blood in small vessels may conceivably diminish venous return and become deleterious to the animal. It is interesting to note that histamine, a vasodilator probably causing increase in small vessel blood volume, decreases the F_{cells} value in splenectomized dogs(9).

2. Blood volume after hemorrhage. The agreement between posthemorrhage cell volume directly determined (CV in Table I) and that calculated by difference (CV′) validates the use of the latter. When there is a change of F_{cells} factor following hemorrhage, posthemorrhage plasma volume cannot be calculated from the posthemorrhage cell volume and hematocrit, as was done in the splenectomized dogs(5). In the present study, posthemorrhage plasma volume is always measured with T-1824. If replacement of plasma proteins is negligible within 2 hours after hemorrhage, the posthemorrhage plasma volume can also be calculated from changes in plasma protein concentrations(5):

$$PV_{2pp} = \frac{PV_1 \times (P.P.)_1 - \text{total plasma protein removed}}{(P.P.)_2}$$

where PV_1 is control plasma volume; $(P.P.)_1$ and $(P.P.)_2$ are respectively control and posthemorrhage plasma protein concentrations. In Table II, PV_{2pp} are thus estimated and compared with $PV_{2T\text{-}1824}$ directly measured with the dye. The results are generally in agreement, and there is no systemic difference. Therefore, when posthemorrhage plasma volume is not directly determined in sympathec-

TABLE II. Calculation of Posthemorrhage Plasma Volume from Changes in Protein Concentration.

Dog No. and sex	Date of exp.	% BV remaining	PV_1 (ml)	$(P.P.)_1$ (g %)	Total $(P.P.)_1$ (g)	P.P. out (g)	Total $(P.P.)_2$ (g)	$(P.P.)_2$ (g %)	PV_{2pp} (ml)	$PV_{2T\text{-}1824}$ (ml)	% dev.*
14 ♀	4/24/59	80.8	391	6.16	24.09	5.01	19.08	5.66	337	340	− .9
13 ♂	4/22/59	77.4	545	6.31	34.39	8.22	26.17	5.61	466	441	+5.7
10 ♀	4/17/59	77.1	556	6.36	35.36	9.76	25.60	5.20	492	467	+5.4
11 ♀	8/15/58	75.7	377	5.35	20.17	5.83	14.34	4.29	334	318	+5.0
8 ♂	3/24/59	75.4	447	5.66	25.30	6.56	18.74	5.30	354	372	−4.8
9 ♂	8/25/58	74.9	604	6.42	38.78	9.67	29.11	5.91	493	472	+4.4
12 ♂	8/27/58	74.1	682	6.62	45.15	13.99	31.16	5.81	536	542	−1.1
9 ♂	7/31/58	73.9	583	6.56	38.24	9.80	28.44	5.96	477	448	+6.5
17 ♀	8/ 5/59	73.6	363	5.15	18.69	5.23	13.46	4.60	293	286	+2.2
10 ♀	8/13/58	73.5	547	5.96	32.60	10.68	21.92	4.95	443	450	−1.6
18 ♀	8/13/59	73.4	538	5.30	28.51	7.98	20.53	4.60	446	420	+6.2
15 ♀	5/15/59	73.0	608	6.26	38.06	13.37	24.69	5.25	470	505	−6.8
7 ♂	8/ 4/58	72.8	647	6.31	40.83	11.89	28.94	5.45	531	505	+5.1
10 ♀	8/29/58	68.6	636	5.81	36.95	11.72	25.23	5.20	485	454	+6.8
11 ♀	11/10/58	66.2	386	5.76	22.23	8.57	13.66	5.05	270	284	−4.9

$$* \ \% \text{ deviation} = \frac{PV_{2pp} - PV_{2T\text{-}1824}}{PV_{2T\text{-}1824}} \times 100.$$

tomized-splenectomized dogs, it is better estimated from changes in plasma protein concentration than from cell volume and hematocrit.

Summary. In 6 out of 25 experiments on sympathectomized - splenectomized dogs, the posthemorrhage F_{cells} factor decreases to below the control range. The constancy of F_{cells} factor following hemorrhage in splenectomized dogs is partially due to sympathetic activity.

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Received July 6, 1960. P.S.E.B.M., 1960, v104.

Determination of N,N′-Diphenyl-p-Phenylenediamine in Animal Tissues.* (25971)

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Interest in the physiological activity of N,N′-diphenyl-p-phenylenediamine (DPPD) stems from its ability to substitute for Vit. E in prevention and cure of tocopherol deficiency diseases, including resorption gestation and muscular dystrophy(1,2). That this effect is not due to conservation of tocopherols in the tissues is evident from the fact that rate of decline of Vit. E in the liver of animals fed a diet deficient in the vitamin is not affected by DPPD administration, and that the antioxidant has curative as well as preventive activity(2). Following administration of d-α-tocopherol-5-methyl-C^{14} to rats, most of the radioactivity in the liver is present in the mitochondria as α-tocopherol, the remaining

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* This investigation was supported by grant from Muscular Dystrophy Assn. of America.

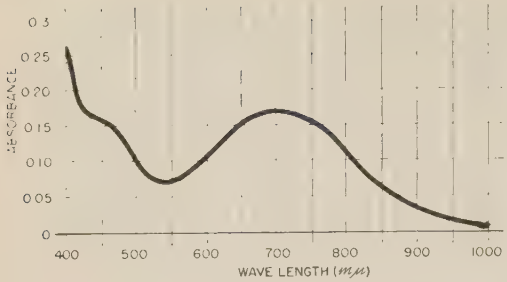

FIG. 1. Absorbance in ethanol of the product of oxidation of DPPD with ferric chloride.

counts being located primarily in the microsomes as unidentified metabolites(3). In view of the apparent nutritional interchangeability of Vit. E and DPPD, the present study was designed to determine the extent to which DPPD accumulates in the tissues and to compare its intracellular distribution with that of α-tocopherol. A new procedure was developed for estimation of DPPD in tissues, based on spectrophotometric measurement of the bluish green color produced by mild oxidation with $FeCl_3$. This method affords greater sensitivity than the semiquantitative procedure used by Pudelkiewicz and coworkers(4) for estimation of DPPD in avian tissues.

Chemical method. Solutions of DPPD in organic solvents are slowly oxidized by atmospheric oxygen to produce a color which ranges, depending upon concentration, from a light yellowish brown to a deep reddish brown. The oxidation is strongly catalyzed by heat and light, and cannot be reversed by sodium bisulfite. Oxidation with H_2O_2 or $FeCl_3$ leads to formation of a bluish green intermediate which can be bleached with sodium bisulfite or which, upon standing, further oxidizes to form the brown product. When the reaction is carried out in ether or acetone the existence of the intermediate is transitory but in ethanol, at room temperature and in dim light, the green color is stable for at least 15 minutes. The absorption curve for this compound, determined using the Beckman DU spectrophotometer, is shown in Fig. 1. A standard curve, based on absorbance at 700 mμ ($E^{1\%}_{1\,cm} = 224$), followed the Beer-Lambert Law and permitted estimation of DPPD over a range of from 6 γ to at least 180 γ/3 ml sample. Sample measurements were made in the above instrument after addition of 1.0 ml of a solution of ethanolic $FeCl_3$ (0.1 g/100 ml) to the sample (or a suitable aliquot) in 2 ml absolute ethanol. *Preparation of samples.* Acetone rather than ethanol was used for extraction of animal tissues because of the greater solubility of DPPD in acetone and the greater ease with which lipids could be removed subsequently by crystallization. Liver samples (about 10 g) were ground with anhydrous Na_2SO_4 until dry and extracted overnight with acetone in darkness at room temperature on a mechanical shaker. Skeletal muscle samples were ground with a mixture of Na_2SO_4 and acid-washed sand. Saponification of fat samples with alcoholic KOH in presence of DPPD resulted in partial oxidation of DPPD, even in presence of pyrogallol and in darkness(5). Consequently the lipids were removed by low-temperature crystallization. After filtering the acetone extracts through No. 1 Whatman paper and washing, volume was reduced to 30-40 ml by vacuum distillation at 40°C under N_2 and the lipids were crystallized by placing the extracts in a dry ice-acetone bath at −70°C for 30 minutes. The lipids were removed by gentle vacuum filtration through No. 1 Whatman paper using a funnel surrounded by a dry ice-acetone jacket. The residue was washed 5 times with 10 ml precooled acetone and the filtrate was evaporated *in vacuo* under N_2 at 40°C, the residue being dissolved in a suitable volume of ethanol. The reaction mixture consisted of 2 ml of this ethanolic solution to which was added 1 ml of $FeCl_3$ solution. Optical density of the sample was read against a reagent blank at 700 mμ. Some samples contained small amounts of yellow pigments which necessitated the use of a sample blank. Known quantities of DPPD added to lard were recovered quantitatively by the above procedure. *Animal experiment.* To determine the degree to which DPPD is accumulated in the liver and skeletal muscle of rats fed different levels of this compound, 3 groups of 3 rats each, weighing about 350 g, were maintained for 7 days on a commercial stock diet containing .005%, .05% or .5% DPPD. After sacrifice, livers and samples of thigh muscle were removed and prepared according to the procedure outlined. *Cell fractionation experiment.* Subcellular distribution of DPPD was determined

TABLE I. Concentration of DPPD in Liver and Skeletal Muscle of Rats Receiving Different Dietary Levels (3 Rats/Group).

Conc. in diet, %	Conc. in tissues, γ/g fresh wt Liver	 Skeletal muscle
.005	4.1- 4.5	2.4-2.5
.05	8.9- 9.3	4.3-4.9
.5	11.0-13.9	7.7-8.0

using livers from 3 rats which had been fed a commercial stock diet containing .05% DPPD for 7 days. Livers were removed under ether anesthesia and homogenized in 9 volumes of 0.25 M sucrose using a smooth-walled glass homogenizer and serrated Teflon pestle attached to a drill press. Cell fractions were prepared in a Servall refrigerated centrifuge essentially according to the procedure of Schneider(6) except that the microsomal fraction was prepared by centrifuging at 25,000 g for 90 minutes. The mitochondrial and microsomal fractions were dried and extracted as described for liver whereas the supernatant was lyophilized before extraction. The extracted residues were analyzed for nitrogen by the microKjeldahl procedure(7).

Results. The data shown in Table I indicate concentrations of DPPD in liver and muscle which result from feeding 3 different levels in the diet. An increase of 100-fold in dietary intake induced only a 3-fold increase in tissue concentration. The inefficiency with which this compound is absorbed from the intestine(8) probably accounts for its failure to accumulate in greater amounts in the tissues. Nevertheless, intakes of .005% or lower are best suited to maintaining reproductivity on a tocopherol-deficient regimen owing to the high order of chronic toxicity exhibited by DPPD for the pregnant female rat(1).

The intracellular distribution of DPPD, based on total recovery in the 3 fractions shown, is given in Table II. Over half was recovered in the supernatant, about one-quarter in the microsomal fraction and a small portion in the mitochondria. It is of interest to compare these results with those for intracellular distribution of lipids. With due allowances for differences in the method used for preparation of cell fractions, the data of Clement and coworkers(9) depict a generally similar distribution pattern for total fatty acid esters as well as for various lipid fractions. These results indicate that distribution of DPPD may be determined largely by its solubility in intracellular lipid.

It is also of interest to compare these findings with those for distribution of the natural antioxidant α-tocopherol (Table II). After administration of C^{14}-labelled d-α-tocopherol to rats, about 60% of the radioactivity in the liver is located in the mitochondria, about half as much being present in the microsomes and only a small fraction in the supernatant. Moreover, the counts recoverable from mitochondria are due primarily to free α-tocopherol whereas the labelled compounds in the other fractions are predominantly metabolites(3). Thus, in contrast to DPPD, the distribution of which appears to reflect distribution of total lipid, α-tocopherol apparently is concentrated in the mitochondria. The implications of this observation in regard to the demonstrated ability of DPPD to substitute for Vit. E *in vivo*, and for the metabolic role of α-tocopherol, are not clear.

Summary. In connection with a study of replacement of Vit. E *in vivo* by certain synthetic antioxidants, liver uptake and intracellular distribution of the Vit. E-active compound N,N′ - diphenyl - p - phenylenediamine (DPPD), following its administration to rats, has been determined by a method based on spectrophotometric measurement of the color developed by oxidation with ferric chloride in ethanol. Over half of the DPPD recovered was present in the soluble fraction of liver

TABLE II. Subcellular Distribution of DPPD in Rat Liver.

	Mitochondrial fraction		Microsomal fraction		Supernatant	
	Range	Avg	Range	Avg	Range	Avg
DPPD content (basis 3 livers), γ	17.6-21.7	19.3	31.5-45.1	36.8	61.4-84.3	74.3
DPPD distribution, %	13.8-15.6	14.8	23.5-31.3	28.2	53.6-62.7	57.0
C^{14} distribution after C^{14}-d-α-tocopherol administration (3), %		60.2		32.5		7.3

cells, one-quarter was associated with microsomes, and a small fraction recovered from mitochondria. This distribution pattern is similar to that reported for total lipids, and indicates that the intracellular location of DPPD may be passively determined by its lipid solubility. Alpha-tocopherol, on the other hand, was found predominantly in mitochondria.

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Received May 27, 1960. P.S.E.B.M., 1960, v104.

Water Diuresis Stop Flow Analysis in the Dog.* (25972)

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When the technic of stop flow analysis was described(2,3) it appeared to offer a means to evaluate more fully the mechanism for control of urea excretion by the kidney. Our data from urea stop flow analyses performed during osmotic diuresis were difficult to interpret. Accordingly, we modified the method using water diuresis. This did not aid in interpretation of the urea data but did provide interesting findings concerning stop flow analysis. During water diuresis stop flow analyses the sodium U/P minimum appeared near the peak of the para-aminohippurate (PAH) curve and potassium and osmolal U/P maxima were distal to the sodium U/P minimum.

Method. The following procedure was found successful in producing water diuresis with rates of urine flow of 6 to 11 ml/min./kidney in the pentobarbital anesthetized dog. Alcohol was administered to the dog in drinking water or by intravenous route. The dog was then anesthetized with pentobarbital. Four hundred ml water were administered by gavage. An infusion of 0.4% NaCl and 2% glucose in water was administered by intravenous route with 10 g alcohol in each liter of infusion. The left ureter was cannulated avoiding the use of cautery. After diuresis had become brisk PAH and creatinine were added to the infusion. When rate of urine flow had become stable and greater than 6 ml/min./kidney the control clearance collections were begun.

Three 10 minute control clearance urine collections were obtained before, and 2 after, the stop flow procedure. The ureteral cannula was occluded for 8 minutes with inulin given by intravenous route 2 minutes before release of the occlusion. Upon release of the occlusion rapid serial collections were obtained from the ureteral cannula for a period of 3 minutes. Heparinized arterial blood samples were obtained at the mid-point of each control clearance urine collection and at time of occlusion, release of occlusion, and termination of the stop flow serial collections.

Osmolalities were performed with a microthermister using the Fiske osmometer. Determinations were made on urine and plasma

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* Supported by grants from U.S.P.H.S. and Alabama Heart Assn. Presented at meeting of Am. Physiol. Soc., Urbana, Ill., Sept. 10, 1959(1).

samples for concentration of inulin using Schreiner's modification of the resorcinol method(4), of creatinine using Hare's method (5) without Lloyd's reagent, of PAH using Selkurt's method with ethylenediamine dihydrochloride(6), and of sodium and potassium using a flame photometer.

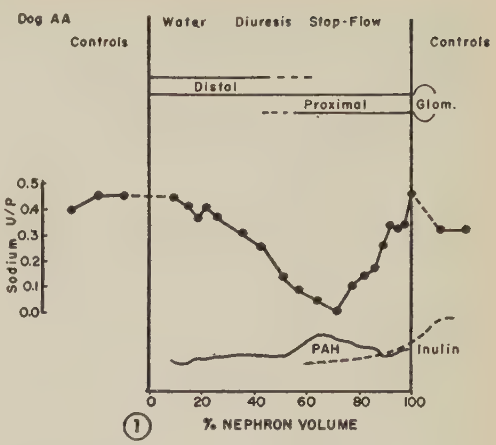
When osmolalities were performed the volume of the sample remaining was too small for all analytical procedures to be carried out on the same animal. Accordingly, 19 dogs were studied and selected comparisons were made. Some of the dogs were given one liter of saline the day before the study.

Water diuresis was considered to be established when the osmolal U/P ratio was well below 1.0. New filtrate was considered to be attained when inulin concentration reached 50% of the inulin peak in the serial stop flow collection samples(3).

Results. Creatinine U/P in the distal nephron collections increased as much as 5- to 7-fold above the free flow creatinine U/P, as opposed to 1.5- to 3-fold increase during osmotic diuresis stop flow. PAH concentrations in the serial stop flow urine collection samples were corrected to the creatinine concentration to construct the curve of the PAH concentration peak in the stop flow samples. This was done with the assumption that the increased creatinine concentration in the distal nephron samples was secondary to water removal.

The lowest sodium U/P ratio was found to occur near the PAH concentration peak (Fig. 1). Maximum potassium U/P occurred distal to the para-aminohippurate peak and the lowest sodium U/P (Fig. 2).

During water diuresis stop flow analysis there occurred demonstrable changes in osmolal U/P in the stop flow samples. Osmolal U/P increased in the distal nephron samples to as high as 1.25. In Fig. 3 data are presented from a dog without saline administered before the study showing the peak of the osmolal U/P to occur distal to the PAH peak and the sodium U/P minimum. The sodium U/P was lower than in the dogs that had received saline illustrated in Fig. 1 and 2. Changes in sodium U/P ratio in the serial stop flow urine collections were accordingly less pronounced.



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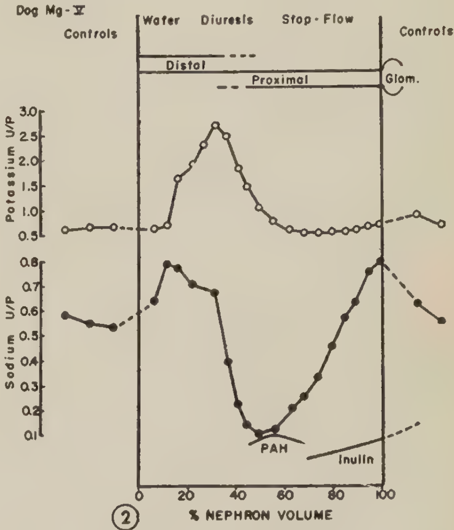

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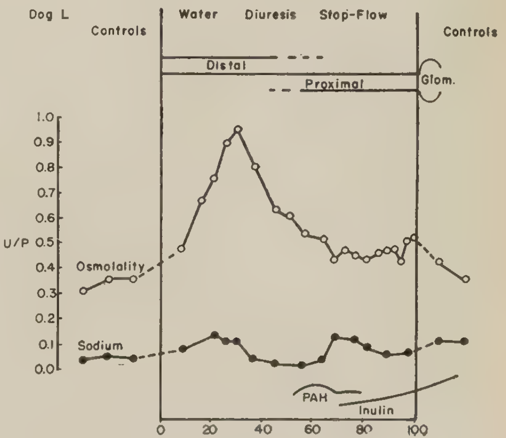

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FIG. 1-3.

The nephron volume, defined as volume of serial stop flow urines obtained prior to appearance of new filtrate, was 8 to 15 ml during water diuresis stop flow as compared to 15 to 24 ml during osmotic diuresis stop flow analyses in our laboratory.

Discussion. The apparently large volume of filtrate reabsorbed during occlusion made interpretation of the data difficult. When a large volume of filtrate is reabsorbed and replacement by filtration is increased it is possible that the PAH peak might be "smeared" down the nephron to the site of the distal nephron. If this were the case PAH would not function as a marker for proximal nephron collections. In accord with the smaller nephron volume during water diuresis stop flow analyses, volumes of urine obtained prior to appearance of the PAH peak and the peak of the potassium U/P were less than found during osmotic diuresis stop flow analyses. Thus, if "smearing" occurred down the nephron it occurred in the distal as well as the proximal nephron and would not explain the presence of the sodium U/P minimum occurring near the PAH peak.

It would be conceivable that during water diuresis stop flow analysis there was a smaller mass of filtrate in the proximal nephron and sodium concentration could be decreased to a greater degree than could occur by removal of the same quantity of sodium from the larger mass of filtrate present in the proximal nephron(7) during osmotic diuresis stop flow. Against this explanation was the occurrence of the PAH peak near the mid-point of nephron volume.

A third possibility would be that active sodium reabsorption did occur in the proximal nephron and was demonstrable during water diuresis stop flow analysis but during osmotic diuresis stop flow analysis was masked by the heavy osmotic load.

The occurrence of the increase in osmolal U/P in the distal nephron demonstrated the distal removal of water in absence of antidiuretic hormone.

The differences found in osmolal U/P and sodium U/P curves in osmotic diuresis and water diuresis stop flow analyses amplified the problems encountered in interpretation of stop flow analysis data.

Summary. During water diuresis stop flow analysis the nephron volume is less than during osmotic diuresis stop flow analysis, there is a demonstrable increase in osmolal U/P in the distal nephron samples, and sodium U/P minimum occurs near the PAH peak. During water diuresis stop flow analysis active proximal reabsorption of sodium is demonstrable or para-aminohippurate is not a valid marker of proximal nephron samples.

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Received December 10, 1959. P.S.E.B.M., 1960, v104.

Effect of Hypotension on K Excretion in the Dog.* (25973)

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Infusion of K salts following oral administration of KCl, raises the clearance of K (C_K) above the filtration clearance(1). This demonstrates tubular secretion of K and it has been assumed that this process accounts for urinary K, with all the filtered K having been absorbed(2). According to this idea, glomerular filtration would have a minimal influence

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* Supported in part by grant from Wisconsin Alumni Research Fn.

on excretion of K. Our initial objective was to alter filtration by reduction of pressure in the renal artery in 2 groups of dogs. One group had ingested only the usual K content of drinking water and a commercial dog food. In another group, C_K was presumed to be much greater, because of prior daily administration of 10 g of KCl in addition to that of the diet and infusion of KCl during the experiment. Data had been obtained on 10 dogs when a similar study appeared(3).

Methods. Male and female dogs, anesthetized with pentobarbital, were used. The ureters were catheterized with polyethylene tubing. Left femoral arterial pressure was recorded by proper connection of the artery with a Hg manometer. With minimal trauma, an adjustable clamp was placed around the aorta. With this a partial compression of the aorta of varying degrees could be effected. Periods of clamping alternated with no clamping. At termination, it was found that the clamp was between the origins of the 2 renal arteries in 11 dogs. The origins were so close that both arteries were affected in 6 and in a very large dog the clamp was caudal to both renal arteries. In 8 dogs 5 g KCl were given orally twice daily for several days before experiment and KCl was infused (.5 meq/min) during experiment. 0.9% NaCl was infused (2 ml/min) during preparatory surgery and with addition of mannitol to 2% through clearance periods. To obtain extraction ratio (E_{PAH}) the left renal vein was catheterized *via* the jugular. Clearance periods were consecutive and nearly always at least 30 minutes in length. Creatinine and PAH were infused at a constant rate and determined by the usual methods. Na and K were determined by the Beckman flame photometer(4).

Results. Effect of hypotension in dogs which had not received a supplement of KCl is typically demonstrated in Table I. Circulation and clearances were maintained during this long experiment more effectively than in some of the other dogs. Urine flow, C_K, and C_{cr} fell with reduction in left renal arterial pressure. The ratio C_K/C_{cr} fell, indicating clearance of K fell more than filtration rate. In 5 other dogs, this type of experiment gave very similar results. The effects were unilateral. When femoral mean arterial pressure (MAP) was lowered to 40-45 mm Hg, C_K fell to 6.9-17% of the pre-clamped period, at 50 mm C_K fell to 24-53% and at 65-75 mm C_K was 44-61% of the pre-clamped period. In one period, about 7 hrs after initial dose of pento-barbital, MAP of 65-70 mm lowered C_K to 18%. Period 7 of Table I was about 7 hrs after initial dose of pento-barbital and although C_{cr} had fallen to 70 and 82% for the left and right respectively of period 1, C_K/C_{cr} was essentially unchanged, indicating a parallelism of filtration and K excretion. By contrast, in the dog with the clamp distal to both renal arteries, partial clamping had no effect on C_K or C_{cr}.

TABLE I. Hypotension and Clearance of Potassium. Wt 15.3 kg.

Kidney	Period	Uv	C_K	C_{cr}	C_K/C_{cr}	Femoral mean pressure, mm Hg
		ml/min.				
L	1	1.6	7.8	21.7	.36	110
R	1	1.5	7.4	22.0	.34	
			Aorta clamped			
L	2	1.0	2.7	17.3	.16	50
R	2	1.4	6.2	22.4	.28	
L	3	1.6	5.7	19.4	.29	130
R	3	1.8	6.8	21.4	.31	
			Aorta clamped			
L	4	.6	.9	10.3	.09	45
R	4	1.8	6.3	22.9	.28	
L	5	1.3	6.6	16.7	.39	115
R	5	1.7	8.4	21.7	.39	
			Aorta clamped			
L	6	.4	.5	6.9	.08	45
R	6	1.2	6.7	20.9	.32	
L	7	1.0	5.6	15.1	.37	120
R	7	1.4	6.9	18.2	.38	
			Aorta clamped			
L	8	.7	2.5	10.9	.23	75
R	8	.9	3.3	15.0	.22	

Effect of hypotension in dogs which had received supplemental KCl orally and intravenously is well illustrated by Table II. The origins of the 2 renal arteries were so close that both kidneys were affected. Although renal excretory capacity for K was much greater than that of the former group, partial clamping of the aorta markedly reduced C_K,

TABLE II. Hypotension and Clearance of Potassium with Administration of Potassium. Wt 14.3 kg.

Kidney	Period	Uv	C_K	C_{cr}	C_K/C_{cr}	E_{PAH}	Femoral mean pressure, mm Hg	C_{PAH}, ml/min.	FF
		ml/min.							
L	1	1.2	20.3	27.5	.74	0.83	105–110	52.1	.53
R	1	1.0	18.1	27.6	.66			54.3	.51
Aorta clamped									
L	2	.7	7.4	15.1	.49	0.86	70	34.8	.43
R	2	.5	3.7	11.2	.33			33.7	.33
L	3	2.5	24.8	18.3	1.35	0.81	100–110	51.1	.34
R	3	1.8	17.7	17.2	1.03			50.0	.32
Aorta clamped									
L	4	.7	4.0	7.4	.54	0.83	60–65	24.2	.32
R	4	1.0	5.9	8.5	.69			29.2	.29
L	5	2.7	19.7	12.8	1.54	0.81	100–130	41.9	.30
R	5	2.7	19.4	13.1	1.48		90–135	38.6	.34
Aorta clamped									
L	6	.3	1.6	3.1	.52	0.78	50–55	10.3	.30
R	6	.3	1.9	3.2	.59			10.3	.31

C_K/C_{cr} and C_{cr}. It is significant that during periods 3 and 5 C_K/C_{cr} was considerably above one. In 5 other dogs, pre-treated with KCl, hypotension had a similar effect upon K excretion, but only in 2 did C_K/C_{cr} during the control periods rise above 1.0 and in one it reached 0.81 and in another 0.65. In 2 of these, the effect was unilateral.

Renal plasma flow, as calculated from data of Table II, was reduced to 64, 46 and 25% of preceding control flow in periods 2, 4 and 6 respectively for the left kidney. FF dropped in period 2 but was essentially unchanged in later periods of hypotension and a similar result was obtained in 2 other dogs. E_{PAH} was determined in 3 dogs and in no case was it changed by the hypotension, indicating that this cellular mechanism was unimpaired.

For 3 dogs renal plasma flow (RPF) of the left kidney could be calculated from C_{PAH}/E_{PAH}. With time RPF fell, although femoral MAP approximated or was slightly higher. In one dog at 4 hrs after initial dose of pento-barbital RPF was 87% of the first period but MAP was 5 to 20 mm higher. In a second dog 6 hrs after pento-barbital RPF was 73% of the first period but MAP was about 20 mm higher. In the dog of Table II, 6 hrs after pento-barbital RPF was 82% of first period and MAP was essentially that of the first period. The reduced renal flow therefore indicates increased vascular resistance in the kidney. Of 8 periods of partial clamping, in these 3 dogs, reactive hyperemia probably did not occur after the clamping since RPF increased only 10% in one period and decreased in the others, in comparison to the pre-clamped periods. For these 3 dogs the K delivered to the left kidney was calculated as the product of RPF and concentration of K in the plasma. The fraction of K delivered to the left kidney, that was excreted, fell greatly during the clamped periods except in one case when it rose. Therefore a reduction in supply of K to the kidney could not wholly account for the effect of the clamping.

Davidson *et al.*(3) reported that lowering of glomerular filtration by compression of a renal artery decreased K excretion. When high rates of Na excretion were maintained by administration of mercurials, sodium sulfate or Diamox®, rate of K excretion was maintained. We had found that Na was almost absent from the urine during the clamped periods, in confirmation of Selkurt(5) and that at MAP of 75 to 80 mm Hg, C_{cr} was not changed but Na excretion was reduced to 40 to 22% of the preceding period. The effect of increased Na excretion on K excretion during hypotension was tested in 7 dogs. Solutions of 5 or 10% NaCl, Na para-amino-hip-

TABLE III. Hypotension and Clearance of Potassium with Infusion of Na Ferrocyanide and KCl. Wt 18.8 kg.

Kidney	Period	Uv	C_K	C_{cr}	C_K/C_{cr}	Femoral mean pressure, mm Hg	Na excretion, meq/min.
		ml/min.					
Aorta clamped. No infusion of ferrocyanide							
L	1	.5	8.2	24.5	.33	55	.006
R	1	.4	5.6	23.9	.23		.004
L	2	3.0	36.6	30.5	1.20	100–110	.181
R	2	3.0	36.0	30.0	1.20		.170
Aorta clamped							
L	3	.9	13.2	25.1	.52	50–55	.061
R	3	1.2	14.5	26.7	.54		.109
L	4	.9	27.7	23.2	1.19	90–95	.067
R	4	.9	25.9	21.8	1.18		.072
Aorta clamped							
L	5	.25	8.1	5.7	1.42	50–55	.019
R	5	.1	7.1	2.5	2.8		.007
L	6	1.0	23.5	16.0	1.47	85–145	.105
R	6	.9	20.0	13.8	1.45		.089
Aorta clamped							
L	7	.9	18.6	13.8	1.34	50–55	.124
R	7	.7	16.1	11.0	1.46		.093
						100–110	

Ferrocyanide infusion during all periods except first.
Priming, 12.5 mmol. Sustaining, 300 μmol/min.

purate or Na ferrocyanide were infused to promote Na excretion. Table III illustrates one of the 2 more successful experiments in maintaining greater Na excretion during clamping and C_K/C_{cr} of 1 or more during the clamping. This dog was pre-treated with KCl orally. However, C_K was greatly reduced by the hypotension. In 14 periods of clamping in 6 dogs, with a high rate of Na infusion and clamping sufficient to drop C_{cr}, C_K ranged from 17 to 70% of the pre-or post-clamped period. In a 7th dog, C_K and C_{cr} were unchanged by clamping but Na excretion fell to 40% of pre-clamping. It was clearly demonstrated in one dog that during 2 periods of clamping, in the one in which C_K fell the least, Na excretion was 3 fold greater.

Discussion. The question arises as to how hypotension and reduced rate of glomerular filtration lowered C_K and the ratio C_K/C_{cr}. In this condition urine becomes almost free of Na and since secretion of K involves an exchange with Na in distal tubule, a lack of Na may be responsible for the fall of C_K in dogs illustrated by Tables I and II. Davidson considered that high Na excretion nullified the effect of reduced filtration rate on K excretion by its provision of adequate Na for exchange with K. In support of this, is the observation that urinary K fell at a slightly lower rate, when human subjects were being depleted of K by a low K diet, in those with a normal Na intake, than in those with a high Na intake(6). However, in Davidson's experiments Na excretion during clamping of the renal artery, was about 0.6 to 1.27 meq/min, an exceedingly high rate. On the other hand, Anderson and Laragh(7) reported that depletion of Na in dogs did not quantitatively change excretion of K in response to injection of K. Tubular secretion of K was demonstrated in severe Na depletion. In their tables it is seen that Na excretion ranged from .0048 to .0288 meq/min when C_K/C_{cr} was above 1.0. Na excretion of our dogs during the high rate of Na infusion and clamping was in this range and markedly above it in 5 cases, although C_K had been markedly reduced. Therefore, hypotension to the level of a resultant lowered filtration rate must reduce K excretion by some factor, in addition to the lowered Na excretion.

Koch *et al.*(8) decided that when the ratio of K/Na in the urine was that in the plasma, an increased urinary K represented incomplete absorption and when it exceeded that in the plasma, K secretion had occurred. In the 5 dogs in which we obtained a combination of sufficient clamping to lower C_{cr} and an elevated Na excretion, clamping raised urinary K/Na in 13 of 14 experiments, about 2 to 5 fold. These dogs had received KCl orally and intravenously and clamping had less effect on K than Na excretion and made tubular secretion more manifest. Nevertheless, after calculation of the K secreted by the tubule, by Koch's use of the urinary K/Na ratio, the data showed that the drop in urinary K was almost entirely due to less secretion and not to greater absorption.

In the data of Koch, for example, it is seen that during the infusion of KCl and NaCl into a K-fed dog, urinary ratio of K/Na fell from 1.7 to 0.126 and C_K rose from 5 to 38. In our 14 periods of aortic clamping during high infusion rates of Na urinary K/Na ranged from 0.6 to 20. On the other hand, Anderson found after NaCl depletion and during KCl infusion, urine Na was less than 6 μeq/min but C_K was above the filtration clearance, with urine K/Na about 70. Therefore, in a comparison of our results with those of 2 other laboratories on the basis of rate of Na excretion or urinary K/Na ratio hypotension should not have materially changed C_K in the dogs infused with large quantities of Na but since it did another factor in addition to Na must be involved. Under many circumstances in dogs, tubular absorption of chloride is a direct function of filtration rate(9) and perhaps tubular secretion of K is influenced by the filtration rate in other ways than by quantity of Na in the tubule.

Summary. In pento-barbitalized dogs, partial clamping of abdominal aorta, always lowered C_K, C_{cr} and C_K/C_{cr}. For these changes to occur mean arterial pressure had to fall below 75 mm Hg. Extraction ratio of PAH was not lowered by hypotension. The calculated renal plasma flow was markedly reduced and reactive hyperemia probably did not occur after clamping. The fraction of calculated quantity of K delivered to kidney that was excreted was much less during hypotension. Hypotension, insufficient to lower C_{cr}, markedly lowered Na excretion but not C_K. In agreement with Davidson, elevation of rate of Na excretion, reduced the effect of hypotension on C_K and allowed C_K/C_{cr} to rise above one. The need to consider another factor in addition to urinary Na in the effect of hypotension on K excretion is pointed out.

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Received March 1, 1960. P.S.E.B.M., 1960, v104.

Decreased Oxygen Requirement for Growth of Fruit Fly Larvae After Continual Centrifugation.* (25974)

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Different laboratories reported that although increased gravity, as simulated by continual centrifugation, can reduce growth of plants and animals, these organisms can survive and actually demonstrate measurable growth(1-7). As expected, these organisms exhibit certain evidences of adaptation. One such evidence is that centrifugation can sometimes evoke a faster than normal growth. This occurs with very young fruit fly larvae during centrifugation at 500 to 700 times gravity(6), or after 24-hour centrifugation at 2000 to 3000 times gravity(5). It has been suggested that, since increased gravity could cause less food materials to be available for developmental processes the animal compensates in part by increasing the efficiency of processes necessary for development. This adaptation might over-compensate, permitting the faster than normal growth which occurs during moderate centrifugation. If such adaptation is permanent, it might well reflect itself after centrifugation by greater than normal growth rate. Under certain conditions this does occur even though there had been a slower growth in the centrifuge(6). The proposed increase in efficiency could have been masked by gravitational retardation of growth until centrifugation ceased. Should this be the case, experimental larvae, returned to control conditions, would consume less than normal amounts of oxygen to accomplish a given amount of growth. Our purpose is to report that the ratio of oxygen consumption to growth is indeed less for larvae returned to normal gravity after 24 hours continual centrifugation. Nyst studied oxygen consumption of Drosophila larvae(8). He found the rate at 25°C for resting larvae to be .04 mm^3/hour/mg of larvae during exponential growth. The rate was directly proportional to the animal's mass rather than surface area as is so often the case with larger animals. We confirmed that there is the same relation to mass for active larvae at 28°C(9). Oxygen consumption was approximately 0.10 mm^3/hour/mm^3 of larvae. Miller(10) has reported that wheat Coleoptiles exhibit altered oxygen consumption after prolonged centrifugation. Gray's group(11) also reported other changes which Coleoptiles undergo in adaptation to high gravity.

Materials and methods. The wild, red eyed strain of *Drosophila melanogaster* was employed. Newly hatched larvae, with volumes of 0.2 mm^3, were centrifuged 24 hours at 20 or 28°C. After removal from the centrifuge, oxygen consumption of active, growing larvae was measured at appropriate temperatures. They were then permitted to grow 24 hours under control conditions. Larvae were cultured and measured in essentially the same manner as previously described(3,5). They developed in plastic centrifuge tubes which contained a seed-free, banana-agar medium. The growth constant, k, is defined as rate of change in natural logarithm of volume (v). In other words, $k = \frac{\dot{v}}{v} = d(\ln v)/dt$. Volume is computed from measurements of larvae's length and width. Geometric means of volume are employed in computing k. Oxygen consumption measurements were performed in a simple respirometer (Fig. 1). It was prepared by inserting a 0.1 ml pipette into a small test tube. Petroleum jelly sealed the junction. Small volumes of ethyl alcohol served as manometer fluid. Rate of descent of this droplet in the pipette was measured. All but upper portions of pipettes were submerged in constant temperature baths. Values cited are corrected to standard temperature and pressure. Ratio of volume rate of oxygen consumption, $\dot{V}$, to absolute rate of growth, $\dot{v}$, can be employed as an index, I, of oxygen required for a given amount of growth. This

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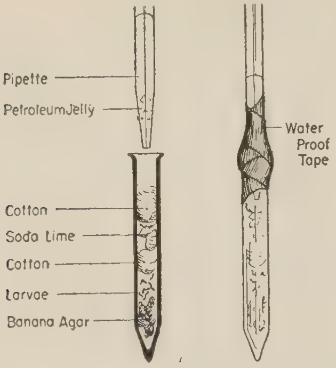
* This work supported in part with grants from Am. Cancer Soc.



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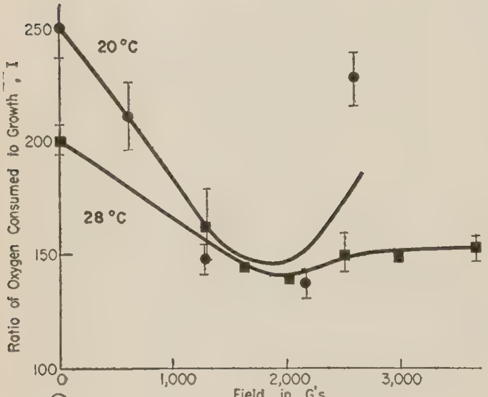

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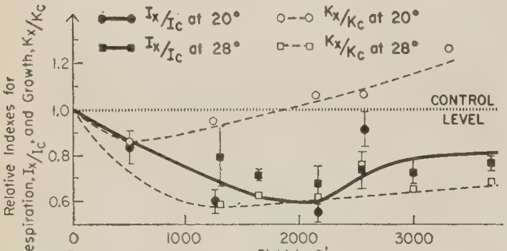

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FIG. 1. Assembly of respirometer. The junction is first surrounded with masking tape to hold petroleum jelly. Approximately 100 one-day-old larvae were necessary to obtain reproducible readings. These respirometers were fashioned from medicine droppers and have a capacity of approximately 3 ml.

FIG. 2. Oxygen consumption relative to growth, I, as a function of the previous centrifugal field intensity. These values of I are for animals after return to control conditions. Values next to the Y-intercept correspond to control values under control conditions, these larvae consume approximately 0.07 ml O_2/hr/ml of larval volume at 20°C and 0.10 at 28°. Vertical lines extending from the points indicate stand. errors. If errors are not shown their magnitude is less than 10.

FIG. 3. Relative oxygen to growth indexes $\frac{I_x}{I_c}$ as a function of gravitational field intensity of previous exposure. The subscript, x, refers to experimental and the subscript, c, to the control. These values were computed upon dividing values in Fig. 2 by control values. Vertical lines extending from points for these values indicate stand. errors. These are not indicated for magnitudes less than .05. Values for relative post-exposure growth constants $\frac{K_x}{K_c}$ are also shown. Their errors, although not shown, would be approximately 10% of the ratio's value. Values for growth constants during centrifugation at these temperatures have been published elsewhere(6).

oxygen to growth index would be equal to $\frac{V}{\dot{v}}$. Since k is defined as equal to $\frac{\dot{v}}{v}$, then the index would also be equal to $\dot{V}/kv$. That formula has been employed in this report. (For this computation, v is equated to arithmetic mean larval volume at time of oxygen measurements.) The index has the advantage of being essentially constant throughout exponential growth(9). It is a unitless quantity which indicates ratio of volume of oxygen consumed to volume of living material produced. Under control conditions, values for I are more reproducible than those for $\dot{V}$, $\dot{V}/v$, or k. Tubes containing experimental larvae were centrifuged in Servall angle centrifuges (Model XL). Centrifuges were enclosed in specially constructed constant-temperature chambers(6). Control tubes were placed in the same chamber. The symbol "G" is used to designate multiples of Earth's gravitational field. Centrifugal fields of from 500 to 3800 G's were employed.

Results. After centrifugation I, oxygen consumption/unit volume of larvae, was below control levels. The oxygen required during a given amount of growth was reduced (Fig. 2 and 3). For larvae centrifuged at 20°C, this was best demonstrated with those exposed to fields from 1200 to 2200 G's. At 20°C, there is an increased efficiency after centrifugation even though there is no marked effect upon growth rate either during(6) or after (Fig. 3) centrifugation. With animals exposed at 28° C, the most significant results were obtained after exposure to fields greater than 1500 G's. No marked difference between relative changes in experimental indexes at the 2 temperatures could be demonstrated for comparable fields (see Fig. 3). During centrifugation at 28°C, the growth constant drops in a more or less linear manner as the field increases beyond 500 G's(6). Such linearity is not demonstrable for I. In all cases, there was little mortality after centrifugation. For both control and experimental cultures, 90% of animals surviving after centrifugation had ceased, were alive 24 hours later.

Discussion. Prolonged centrifugation causes fruit fly larvae to develop altered metabolism. This is evidenced by the fact that, upon return to control conditions, these animals can accomplish more than the normal amount of physical growth with a given quantity of oxygen. The mechanism of this alteration is unknown. It is tempting to interpret this as an increased efficiency of growth processes.

The validity of the preceding suggestion could well depend upon how broadly we wish to define "efficiency" and "energy for growth." Let us define efficiency in terms of the ratio of growth accomplished to food metabolized. Then, assuming a constant, caloric equivalent for oxygen, efficiency has increased.

One would not necessarily expect changes in chemical production of protoplasm to greatly alter the overall oxygen requirements. Processes other than production of protoplasm could be altered. These other processes might include various movements, digestion, and maintenance. Should any change cause a greater efficiency, then, once gravitational stress is removed, a larger fraction of food would be available for growth processes.

After centrifugation, larvae do develop into pupae. However, unlike controls, a sizable percentage of these individuals do not emerge as adults. Perhaps, food materials can be conserved by sacrificing structures.

Summary. 1. Prolonged centrifugation can alter an organism's growth. 2. After centrifugation at 20 and 28°C, fruit fly larvae do not require as much oxygen for growth. 3. It is suggested that the animal has adapted by developing a greater efficiency for growth.

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Received March 7, 1960. P.S.E.B.M., 1960, v104.

Adaptation of Orbital Bleeding Technic to Rapid Serial Blood Studies.

(25975)

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During investigations requiring serial blood samples on experimental animals our attention was drawn to the ingenious but apparently little known eye bleeding procedure (1-5). Tail bleeding is difficult, time consuming and unsatisfactory from the standpoint of contamination of the collected blood. Other methods have obvious drawbacks.

Methods. Since enzyme studies usually require plasma rather than whole blood it is desirable to centrifuge directly in the original blood collection tube. The technic requires a

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* With assistance of David Bardell and Elizabeth Booth.

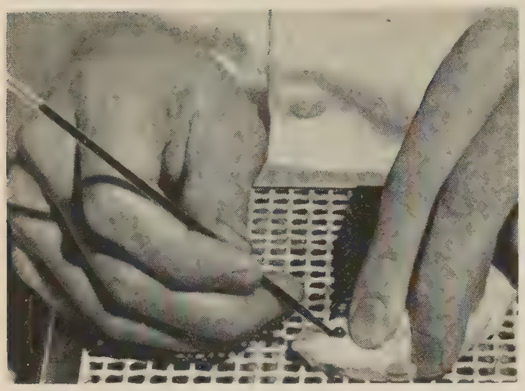


FIG. 1. Proper position of fingers, animal, and pipette during bleeding procedure.

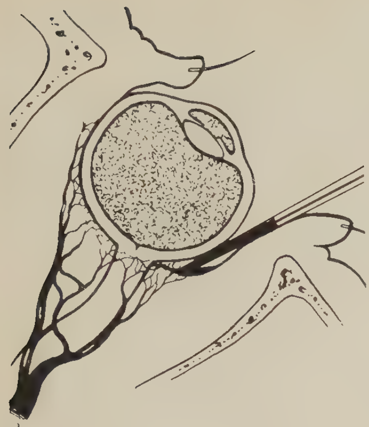
small, disposable, heparinized pipette with polished tip for non-traumatic entry into the orbit alongside the eye. Such a pipette is now commercially available.[†] Utilizing the technics described here, a technician can bleed 100 mice in about an hour and a half. Our standard procedure is as follows: 1. Test tubes, 13 x 100 mm, are labelled with appropriate experimental numbers and are set up in a rack to receive the blood filled capillary tubes. 2. As illustrated in Fig. 1, the donor animal is held by the back of the neck with the left hand, and the loose skin of the head is tightened with thumb and middle finger. With the aid of the index finger, the eye is made to bulge slightly by further traction of the skin adjacent to the eye. The tip of the pipette is then placed at the lower or inner corner of the eye and gently but firmly slid alongside of the eyeball to the ophthalmic venous plexus which lines the back of the orbit (Fig. 2). The venous capillaries forming this network are so fragile that they rupture upon contact with the tip of the pipette and resulting hemorrhage fills the orbital cavity which serves as a useful reservoir. A slight withdrawal of the pipette frees the tip so that the accumulated blood is immediately drawn into the tube by capillary action. The actual bleeding part of the procedure, while the pipette is in position, takes about 2 seconds. Residual blood around the eye is swabbed clear with a soft absorbent tissue to avoid clot formation. Bleeding usually stops immediately upon withdrawal of the pipette and reestablishment of normal ocular pressure on the venous network. By appropriate change in positioning of the animal, either eye may be bled, although the right eye is more convenient for a right-handed technician. 3. If blood is to be centrifuged, it is necessary to seal the upper end of the collection tube, by thrusting the blunt end of the tube through a thin wall of softened clay[‡] which forces 3 to 5 mm of the plastic material into the bore of the tube. This is held in place by a tight fitting serum ampule stopper.[§] 4. The sealed blood pipettes are placed in their previously labelled test tubes, with the stopper end down, for centrifugation. For most purposes, 2,000 rpm (approx. 1,000 x G) for 20 minutes[||] provides adequate separation of plasma and red cells. Either a horizontal or angle head may be employed, although traces of red cells tend to cling to the sides of the tubes with the latter. 5. If the hematocrit (packed red cell volume) is desired as an additional parameter, it may be obtained at this stage by holding the capillary tube against the inclined scale previously described(6). This gives the hematocrit directly in percent. 6. To obtain the plasma, the tube is nicked with a file just above the plasma-erythrocyte interface and is readily snapped in two. The plasma is recovered and measured by touching the tip of a 0.1 ml or 0.025 ml pipette to the open end of the severed tube. Exact adjustment of plasma volume is accomplished by applying a wiping tissue to the tip of the measuring pipette. The collection tube holds approximately 0.2 ml of blood which yields about 0.1 ml of plasma. This is ample for most enzymes determined spectrophotometrically when employing a 1.0 ml microcuvette. If more plasma or blood is required, additional tubes may be obtained from the same donor.

[†] Micro Blood Collection Tubes, Heparinized with Ammonium Heparinate, Scientific Products, Div. of Am. Hospital Supply Corp., Evanston, Ill.

[‡] Children's plastic modeling clay, blue, or green color to contrast with blood cells.

[§] No-2319-B Rubber Stoppers, Arthur H. Thomas Co., Philadelphia, Pa.

[||] International Refrigerated Centrifuge, Model PR-2 Head No. 253.



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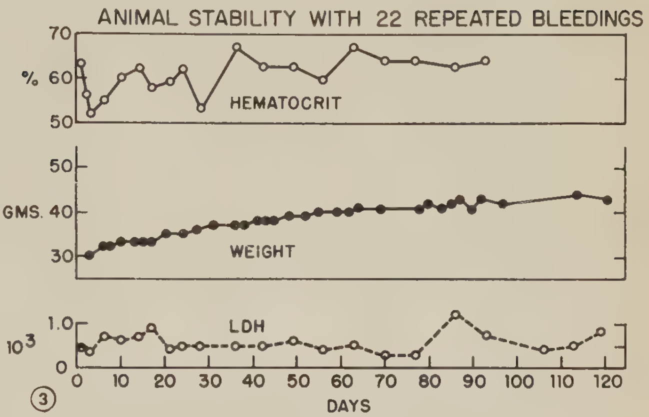

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FIG. 2. Diagrammatic illustration of location of ophthalmic venous plexus and relationship of pipette entry into the orbit.

FIG. 3. Lack of significant influence of repeated 0.2 ml ocular bleedings of mice over a period of 4 mo in respect to hematocrit, mouse wt, and plasma lactic dehydrogenase activity (LDH).

However, it is necessary to bear in mind the results of excessive bleeding on the hematopoietic system, if this is of pertinence to the experiment, since collection tube volume of 0.2 ml may represent 5 to 10% of total mouse blood volume. If large volumes of blood are required, the mouse can be exsanguinated by this technic.

Discussion. Moderate use of this procedure, such as 1 or 2 bleedings per week, seems to be harmless to the animal, and even daily bleedings have been imposed for limited periods with a lowered hematocrit being the only adverse effect noticed. No anesthetic is required with this procedure and, although the technic carries a formidable psychological barrier for an identifying human when it is first observed, neither the eye nor the health of the mouse appears to be damaged. The same mice have been bled repeatedly from both orbits for periods of several months without evidence of blindness or other serious damage. Occasionally accidental trauma or infection may require the bleeding to be carried out on the opposite eye or the animal to be sacrificed. As indices of animal stability, Fig. 3 illustrates uniform level of weight, hematocrit, and a blood enzyme (LDH) in normal mice bled repeatedly during an experiment lasting several months. For further data see(7,8).

While our experience has been mostly with mice, the same technic has been used successfully with a variety of other small laboratory animals. Chickens seem to be an exception, however, since the structure surrounding the eye is too rigid to permit passage of the pipette to the posterior vascular area.

For enzyme and chemical determinations requiring plasma free of hemoglobin and other erythrocyte contents, we have found it desirable to keep the blood cool at least until plasma has been separated from red cells. The rack of collection tubes is therefore placed in a refrigerator or a portable ice box during bleeding and while waiting centrifugation. This seems to minimize interfering subliminal hemolysis. The procedure described here permits daily following of the course of a disease or effects of treatment on individual laboratory animals, as well as groups, similarly to the close clinical observations on a human patient. With this single bleeding operation requiring about 30 seconds, one can obtain a smear for differential cell counts or cytological study, erythrocyte sedimentation rate, hematocrit, and plasma for biochemical determinations or enzyme studies.

Summary. Adaptation of orbital bleeding procedure to rapid, serial blood sampling of mice and other small laboratory animals has been made. Blood (0.2 ml) is collected in disposable, heparinized micropipettes by rup-

turing the fragile opthalmic venous plexus with tip of blood collection tube. The tube may be sealed and centrifuged for hematocrit determination and plasma separation for enzyme or other biochemical studies. Bleeding procedure for a mouse requires less than 30 seconds, and 100 mice can be bled and processed by a technician in about 1½ hours. Appropriate illustrations demonstrate technical details of procedure.

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Received March 7, 1960. P.S.E.B.M., 1960, v104.

Rapidity of Breakdown of Nucleotides in Different Tissues.* (25976)

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Mammalian blood can be incubated *in vitro* for several hours without affecting distribution and amounts of nucleotides in the formed elements of the blood. This has allowed us to perform incorporation studies under essentially *in vivo* conditions. In attempting to perform similar incorporation studies on other mammalian tissues we discovered that after incubation, the major nucleotide, ATP, had largely disappeared and that AMP was now present in considerable quantities. The present paper describes our attempt to determine *in vivo* distribution of nucleotides in certain tissues and the rapidity of change in this distribution after removal of the tissue from the animal.

Materials and methods. Dog muscle and dog and rabbit intestinal mucosa were originally selected because they might be expected to contain large amounts of nucleotides that would be turning over fairly rapidly. The muscle was minced with scissors while the mucosa was merely scraped off the intestine with a glass microscope slide. The nucleotides were extracted from either preparation with cold trichloroacetic acid (TCA) and fractionated on Dowex-1 formate columns according to our previous procedure for blood (1). In the first experiments a dog was given a lethal dose of Nembutal intravenously and the hind legs rapidly skinned. One portion of leg muscle was dropped into ice cold 10% TCA and minced with scissors. A second portion was excised and allowed to stand at room temperature for 20 minutes. It was then treated like the first. A third portion was minced with scissors in calcium-free Krebs-Ringer-Bicarbonate medium(2) containing 200 mg % glucose. Ice cold TCA was then added to the mince. A fourth portion was prepared like the third except that it was incubated 2 hours at 37° under 95%O_2–5%CO_2 before TCA was added. No attempt was made to weigh portions of muscle accurately, because this would have slowed down handling of the tissue and because only distribution of nucleotides was of interest at this point. A second type of experiment was designed to ascertain if nucleotides in intestinal mucosa were as labile upon incubation as nucleotides in dog muscle. For this purpose a dog was injected intravenously with lethal dose of Nembutal and a length of small intestine removed as quickly as possible. This was slit open, rinsed briefly with cold saline, and

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* This work supported by grant from Nat. Inst. of Arthritis and Metab. Dis., N.I.H., Bethesda, Md.

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TABLE I. Adenine Nucleotide Distribution in Tissues.

	μmoles/tissue sample		
	AMP	ADP	ATP
A. Dog Muscle			
Minced immediately in cold TCA	.36	4.98	41.8
Stood 20 min., then minced in cold TCA	1.16	11.72	44.6
Minced in incubation medium, then TCA added	2.59	3.59	1.59
Minced in incubation medium and incubated 2 hr before TCA added	.31	3.52	.26
B. Dog Intestinal Mucosa			
Intestine slit and dropped into cold TCA	1.20	.34	.28
C. Rabbit Intestinal Mucosa			
Intestine slit and dropped into cold TCA	.87	.34	.51
Mucosa scraped off and dropped into cold TCA	1.00	.28	.32
Mucosa scraped into 10 ml of human plasma and incubated 2 hr before TCA added	.13	.08	.08
Idem, except 50 ml of plasma used	.07	.33	.32
TCA inj. into intestine *in vivo*	.39	.68	2.20

dropped into ice cold TCA. A third series was designed to verify lability of nucleotides in intestinal mucosa from another species. In the first experiment a rabbit was shot in the head and a piece of small intestine removed immediately. This was slit and dropped into cold TCA. At the same time, a second piece of intestine was removed and the mucosa scraped off and plunged into cold TCA. A third piece of intestine was scraped and the mucosa suspended in 10 ml of human plasma. A fourth piece was treated like the third except 50 ml of plasma were used. The third and fourth samples were incubated at 37° under 95%O_2–5%CO_2 for 2 hours and then added to cold TCA. Experiments with intestinal mucosa indicated that nucleotides from this tissue were extremely labile, and raised the question as to how one could extract these compounds to avoid deterioration. A possible solution was to inject TCA into living tissue. Accordingly a rabbit was anesthetized with Nembutal and the small intestine exteriorized through slit in the abdomen. A section of intestine was clamped at either end, care being exercised not to disturb blood circulation to this section. Into the lumen of this closed segment of intestine ice cold 10% TCA was rapidly injected. The instantaneous blanching of intestinal wall indicated that TCA had penetrated the mucosal layer.

Results are presented in the Table. From the first series it is apparent that in freshly excised dog muscle there was a relatively large quantity of ATP, some ADP, and very small amounts of AMP. This distribution is not unlike that found in freshly-drawn mammalian blood(1). The second experiment (A.2) suggested that, on standing, some of muscle ATP broke down to ADP and AMP. As is shown in A.3 mincing of dog muscle in incubation medium caused nearly complete loss of ATP. Exp. A.4 indicated that incubation did not lead to restoration of original nucleotide pattern. Dog muscle, like blood, contains other nucleotides than those recorded here, but these are ignored to simplify conclusions of these studies.

The experiment with dog intestinal mucosa (B.1) revealed that ATP was largely decomposed, giving rise to ADP and AMP.

Experiments with rabbit intestinal mucosa indicated the same sort of deterioration of the nucleotide pattern as seen in dog intestinal mucosa. In Exps. C.1 and C.2 tissue nucleotide patterns indicated a preponderance of AMP over ATP. Our experience with other tissue indicated this to be a grossly abnormal finding but at first we could not say with certainty that intestinal mucosa might not have an unusual nucleotide pattern. Our final results in C.5 reinforced our original assumption, *i.e.,* that ATP far exceeds ADP or AMP in *in vivo* conditions. Exps. C.3 and C.4 demonstrated that nucleotide pattern in intestinal mucosa would not revert to normal under the incubation procedures used here.

In Exp. C.4 with 50 ml of plasma the ATP level was preserved at a slightly higher level than in Exp. C.3 in which only 10 ml of plasma were used. This suggests that although these incubation conditions were far from ideal, the extra plasma did have some beneficial effect, perhaps by supplying a nutrient or neutralizing some toxic substance.

Discussion. Our earlier studies showed that whole blood could be incubated for several hours with no loss of ATP. Extension of these studies to other tissues was uniformly unsuccessful. In dog muscle, mincing in incubation medium caused nucleotide breakdown. In dog or rabbit intestinal mucosa, the cells could not even be scraped off into any kind of medium without deterioration of the nucleotide pattern. Injecting trichloroacetic acid into the living tissue is similar to the study of Dietrich and Friedland(3) which reported *in vivo* injection of perchloric acid into rabbit intestine. Their AMP:ADP:ATP ratios of 0.10:0.31:1.28 were not greatly different from ours, but perhaps both results are only approximations to the true *in vivo* distribution.

Summary. Our experiments indicate that the nucleotides of different tissues vary greatly in lability. When dog muscle was removed rapidly and minced in ice cold trichloroacetic acid, its nucleotide pattern resembled that seen in fresh blood, *i.e.*, ATP greatly exceeded ADP or AMP. Delay in mincing the tissue caused some decrease in ATP with increases in ADP and AMP. On the other hand, dog or rabbit intestinal mucosa was so labile that usual procedure of removing tissue and transferring it immediately to cold trichloroacetic acid was much too slow. Injecting cold trichloroacetic acid into the live tissue was necessary to give a nucleotide pattern which resembled patterns seen in other tissues. Incubation under circumstances tried, were unsuccessful in restoring tissue nucleotide patterns. Extrapolation of foregoing results suggests that studies involving either distribution of nucleotides in tissues or determination of tissue nucleotides after *in vitro* incubation must indicate degree of lability of these tissue nucleotides.

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Received May 13, 1960. P.S.E.B.M., 1960, v104.

Effect of Thyroidectomy on Development of Polyploid Nuclei in Rat Liver.*† (25977)

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The role of growth hormone in the appearance and maintenance of polyploid cells in normal liver was originally described by Helweg-Larsen(1) and Leuchtenberger *et al.*(2) and confirmed and extended by DiStefano and Diermeier(3) and Geschwind *et al.*(4). Recently, involvement of 2 other hormonal systems has become apparent. The gonadal hormones have been implicated as a result of a study of the effect of castration on polyploidization of rat liver(5) and, as indicated by Carriere in preliminary reports(6,7), thyroid hormone is also involved. The role of the latter has been further elaborated upon in recent experiments(8) on effects of thyroxin

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[*] This work supported by U.S.P.H.S. grant.

[†] The authors are grateful to Dr. L. Lapham, Western Reserve Univ. for making available the photometer, and to E. Nagy and M. Henry for valuable technical assistance.

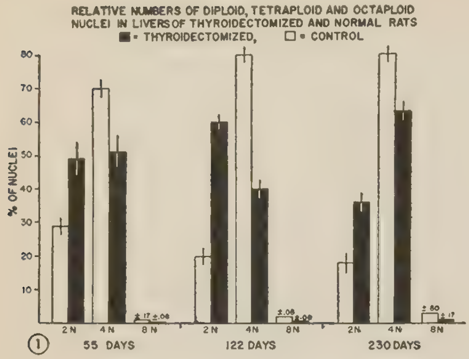
[‡] Student Scholar of U.S.P.H.S. and Kentucky Heart Assn.

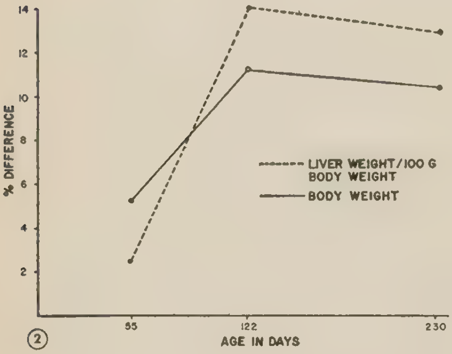
and growth hormone on ploidy development in livers of dwarf mice and hypophysectomized rats. The present study confirms the effect of thyroidectomy on polyploidization of the rat liver and, since it reveals a unique effect of partial thyroid deprivation or its equivalent, extends the observations already made. It also indicates the necessity of extended-time studies when dealing with problems of developmental ploidy in rat liver, and attempts to elaborate upon the mechanics of ploidy as a growth phenomenon. It will also be demonstrated that polyploid composition of the liver appears to be a more sensitive indicator of thyroid deficiency than growth of the animal as proposed by Evans *et al.*(9).

Materials and methods. Sixteen females from 9 litters of Sprague-Dawley rats were thyroidectomized at 21 days of age, 13 unoperated littermates serving as controls. The animals were fed *ad libitum* on a standard Purina diet and 1% calcium gluconate. Weekly body weight determinations were made and tissues were taken at 55, 122, and 230 days of age. At these times total liver weights were obtained for all animals, and tail, body, and humerus lengths for the 122-day group. Blocks of liver were removed from the center of the left lateral lobe, fixed in Lavdowsky's fluid and sectioned at 10 μ. As control on completeness of thyroidectomy, tracheas and adjacent muscle masses were sectioned, and every tenth section examined. A few residual follicles were found in 3 of the animals but they were considered to be completely thyroidectomized since their liver cytology was consistent with that of the other operated animals. Since pituitary acidophil degranulation follows thyroidectomy(10,11), the hypophyses of the 122-day operated and control animals were preserved and stained with aldehyde fuchsin to determine effectiveness of the operation. For the purpose of this paper, binuclearity was ignored, and polyploidy considered only from the standpoint of DNA content per nucleus. Establishment of DNA classes was based on 30 cytophotometric determinations per liver as previously described(5). This information was used as the basis for visual classification of 2000 nuclei per liver, 1000 by each of 2 trained observers working independently on coded slides (5).

Results. Because the animals were maintained on a normal diet we believe that our data represent the result of initial extreme thyroid deficiency followed by either thyroxin replacement from hypertrophying accessory tissue or gradual supplementation by dietary iodide and/or thyroxin. Thus, the recent work of Evans *et al.*(9), indicating that levels of iodide sufficient to keep pituitary acidophils active might conceivably be found in a normal diet, can explain, in part, the results obtained below. While it has been observed that the experimentals, at all age levels, have obviously fewer polyploid nuclei than littermate controls, for 6 of the animals a small percentage of octaploid nuclei were counted by the visual method, while none with the octaploid amount of DNA could be found by cytophotometry. This indicates a certain number of nuclei have increased in volume without DNA increase, and suggests interference with DNA synthesis in the thyroidectomized rats. It also warns against the assumption that nuclear volume is consistently related to degree of ploidy.

Fig. 1 summarizes, by age group, the results of visual classification of polyploid nuclei. These nuclei, absent at birth, increase in number with age so that at time of weaning (day of operation in these experiments) the livers contain 90% diploid (2N), 10% tetraploid (4N), and virtually no octaploid (8N) nuclei. Stability for normal animals is reached by 122 days of age, at which time the ploid composition is approximately 18-20% 2N, 80% 4N, and 1-2% 8N. At 55 days of age formation of ploid nuclei in experimental livers has progressed beyond the level at time of operation, but lags significantly behind the control group. Nevertheless, livers of the thyroidectomized animals are predominantly 4N. At 122 days of age the ploid population has "regressed" so that livers of the experimental group contain only 40% 4N nuclei with a correspondingly smaller percentage of 8N nuclei. Since the livers were growing between 55 and 122 days this "regression" might also be interpreted as a retarded formation of polyploid nuclei during continued production of diploid nuclei. In either event, the liver





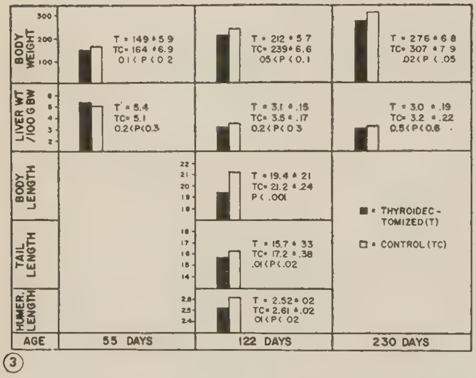


FIG. 1. % composition of polyploid nuclei, by age group, in livers of normal and thyroidectomized rats (operated on at 21 days). Stand. errors indicated by vertical lines.

FIG. 2. % differences between relative liver weights of normal and thyroidectomized rats, and between body weights of the same animals.

FIG. 3. Mean body and relative liver weights, and tail, body and humerus lengths for normal and thyroidectomized rats, arranged by age group. P values determined from t test for small samples.

cell appears to be more sensitive to thyroid deficiency at 122 days than at 55 days, since there is no reason to believe that thyroidectomized animals are any more thyroid-deficient at 122 days than at 55. In fact, as shown below, there is good evidence that they are less thyroid deficient at 122 days than at 55. Alternatively, the polyploid status at 122 days may only reflect a delayed response to a much earlier stimulus, although most of the data gathered thus far would lead one to expect a faster response to hormonal imbalance. At 230 days the situation has again reversed so that the operated animals appear to be approaching the controls cytologically, although there is still a significant difference at all ploid levels.

The polyploid regression appears similar to that following hypophysectomy and, because pituitary acidophil degranulation follows thyroidectomy, can be most satisfactorily explained by diminished growth hormone output. Furthermore, the sequence of events as described above is quite consistent with observed growth patterns and pituitary cytology. Although no counts were made in pituitaries of the 122 day group, we have observed large numbers of granulated acidophils in all specimens. If the assumption can be made that complete acidophil degranulation followed thyroidectomy(10,11) it is evident that extensive acidophil regranulation has occurred in all of the experimental pituitaries. Considering the evidence cited by Geschwind *et al.* (8), reactivation of acidophils in presence of dietary iodide or thyroxin is an acceptable possibility. Since the liver appears to exhibit marked sensitivity to lack of thyroxin at the same age at which regranulated acidophils are observed, appearance of the latter must, for the present, be considered as representing the beginning of acidophil reactivation.

Growth differences for the 3 age groups support the concept of replacement of iodide and/or thyroxin from either dietary or endogenous sources, which is strikingly reflected after 122 days (Fig. 2). For the 2 parameters recorded the differences are least at 55 days, maximum at 122 days, and slightly less at 230 days, indicating a tendency for operated animals to approach the growth of normals at 230 days. While the decrease between 122 and 230 days is slight it is significant that, for each of the litters studied, the change is consistently in the same direction.

Since polyploidization is a liver growth process characteristic of postnatal growth of the organ, we have attempted to analyze upon what other growth factors, if any, the process is dependent. Absolute and relative liver weights and liver growth rate have been examined as possible determinants. At 122 days, the age of maximum liver effect, mean liver weight for operated animals is 6 g, equivalent to mean liver weight of 55 day normal rats. Comparison of ploidy status in the 6 g liver of the 122 day thyroidectomized rat and the 6 g liver of the 55 day normal rat (Fig. 1) reveals that the 2 are dissimilar, and that polyploid percentages in the operated animals are characteristic of normal animals younger than 55 days. Fig. 3 is a summary of mean values of several growth parameters recorded for the 3 age groups. Examination of relative liver weights reveals a decrease with age in normal animals, declining from approximately 13 at birth to about 3.5 from 122 days. Since polyploidization of normal liver occurs coincidentally with decrease in relative weight, it might be assumed that an inverse ratio exists between it and polyploid status. However, examination of P values reveals no significant difference between operated and control relative liver weights at any age level, although polyploid composition shifts significantly (Fig. 1). Furthermore, significant polyploid shifts can still be observed in operated rats once relative liver weights have reached stability. With respect to liver growth rate we have previously shown, in the castrate female, that growth rate of both body and liver is accelerated, while formation of 8N cells is retarded(5). Therefore, it appears that absolute and relative liver weight, and liver growth rate, while apparently coordinated with polyploid development in the normal rat, are not positively related and cannot be considered as determinants for polyploid composition of the liver.

Fig. 3 further reveals that body weight difference between thyroidectomized and control animals becomes increasingly significant with age, and differences in body, tail and humerus lengths are quite significant at 122 days, but not as great as one would expect from animals thyroidectomized and injected with I^{131} or maintained on iodine-free diets. However, since the polyploid shifts approach the expected maximum, the experimental conditions have served to demonstrate that polyploid composition of liver is a more sensitive indicator of thyroid deficiency than the usually recognized growth parameters.

Summary. Thyroidectomy of weanling rats kept under normal dietary conditions results in initial prolonged retardation in production of polyploid nuclei in the liver, followed by gradual resumption of polyploid cell formation over long periods. The effect appears to be mediated, at least in part, through the pituitary, and polyploid shifts are greater than can be explained on the basis of absolute or relative liver weight, or liver growth rate. The liver cytology appears to be more sensitive to thyroid deficiency than the general growth of the animal, the time of greatest sensitivity for this study having been observed at 122 days of age.

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Received May 19, 1960. P.S.E.B.M., 1960, v104

Effect of Reduction of Bowel Flora on Experimental Staphylococcal Infection in Mice.* (25978)

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Reduction in total number of viable organisms in the gastrointestinal tract has been of interest experimentally and clinically for several years. In the laboratory it is possible to raise animals under exceptional conditions and thus disease-free (*i.e.* specific pathogen free) or even germ-free specimens may be obtained. Under clinical circumstances reduction in bowel flora is desirable when surgery is to be performed on the alimentary tract. This is usually achieved by use of an antimicrobial agent, most frequently neomycin.

Dubos and Schaedler(1,2) have demonstrated convincingly that mice reared with their alimentary tracts free of most common Gram negative enteric organisms are more susceptible to staphylococcal infections than their control counterparts. Since the strain used by Dubos and Schaedler is an inbred strain the authors suggest an alternate explanation for this phenomenon, namely, that this increased susceptibility to staphylococcal infection on a hereditary basis cannot be ruled out. It is the purpose of this investigation to determine whether or not reduction in bowel flora *per se* may be the important factor in permitting a more virulent infection to occur.

Materials and methods. Male Swiss Webster albino mice approximately 4 weeks old and weighing 16 to 20 g were housed in the usual steel mesh cages, 10 mice to a cage. As soon as the animals were obtained from the farm they were placed on ground food made by pulverizing commercial mouse pellets to accustom them to powdered food for one week before additives were included in the diet. In the groups of animals that received neomycin the drug was added to the diet by thorough mixing in a food tumbler. The experiments were divided into 4 groups.

Group I. *To evaluate effect of neomycin on intestinal flora of mice*: A control group and 3 dosage levels of neomycin were used, 0.05, 0.1, 0.2% of the diet. The mice were kept on this diet throughout the experiment. The animals were sacrificed periodically and the small intestine (from pylorus to cecum) was removed, homogenized, and an aliquot diluted and plated on agar pour plates. The method has been previously described(3).

Group II. These experiments measured survival of 160 mice. 0.2 cc of a 1:1 dilution of a strain of hemolytic *Staphylococcus aureus* (Giorgio) was given intravenously as the challenge. This strain is susceptible *in vitro* to penicillin concentrations of 3.1 μg/ml. The animals were divided into 4 groups: a control group; a group treated with neomycin (0.1% of diet) before challenge; a third group treated daily from day of challenge with 1.5 mg of aqueous penicillin G intramuscularly for 28 days; and a fourth group that received both penicillin and neomycin as above. Neomycin was added to the diet one hour prior to challenge. Survival was determined in each group.

Group III. These experiments were identical to those in Group II except that after 72 hours the groups of animals receiving neomycin were divided in half, and only one-half in each case continued on neomycin.

Group IV. This experiment was identical to Group II except that the microbial population of staphylococci in the kidneys of 200 mice was computed instead of survival. The technic is that of homogenizing the kidneys, and appropriately diluting and plating an aliquot. Total microbial census can be computed from this procedure(4).

Results. Group I. Oral neomycin significantly lowered the microbial census of the small intestine. In Fig. 1 it may be seen that 0.1% of the diet as neomycin caused the greatest fall in population. After a 72-hour period there was a gradual return of organ-

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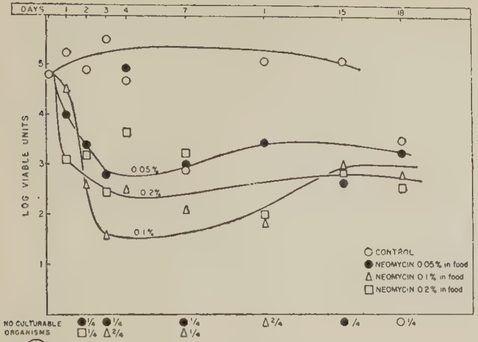
* This study supported in part by contract, Research and Development Division, U. S. Army.



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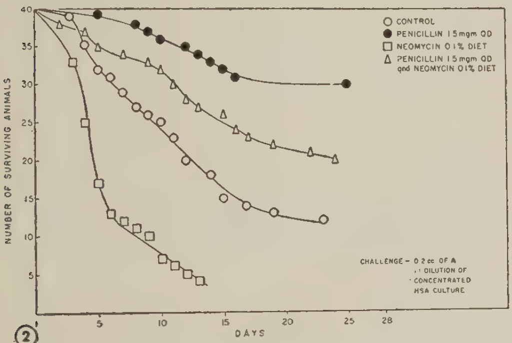

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FIG. 1. Effect of neomycin on total microbial census of the small intestine of normal mice.

FIG. 2. Effect of neomycin bowel preparation on survival of mice challenged with hemolytic *Staphylococcus aureus* (Giorgio) (penicillin resistant) treated with penicillin.

isms. The rise and fall is qualitatively made up of Gram negative organisms, especially *Escherichia coli.* There were no deaths in this group and the animals appeared healthy.

Concentration of neomycin in blood was determined in all sacrificed animals. No neomycin activity could be shown by the tube dilution method using *Sarcina lutea* and hemolytic *Staphylococcus aureus* (Wolbeck) as test organisms. Both these strains are susceptible to neomycin concentration of 0.12 μg/ml. Several groups of mice were maintained on neomycin diet for 4 months. There were no deaths and all animals gained weight.

Neomycin as 0.1% of the diet was used exclusively throughout the remainder of the experiments described.

Group II. In the survival studies there was a definite increased susceptibility to staphylococcal infection of the neomycin-prepared animal as compared to control animals (Fig. 2). The SD-50 for the control was 14 days and for the neomycin group 5 days. Treatment with penicillin increased survival over the control animals when used alone or with neomycin.

Group III. Survival studies indicated that discontinuing neomycin administration after 72 hours has no effect on the outcome. The death rate remains constant and all animals are dead 15 days from time of challenge. The SD-50 of 5 days is the same as in animals continuing on neomycin.

Group IV. Microbial populations in the kidneys were highest in the group receiving neomycin alone. The census was significantly higher than the control animals. In the groups receiving penicillin the populations were lower than the control throughout the 28-day period.

Discussion. It is evident from these results that mice in which the intestinal flora are reduced by chemical means (neomycin) are more susceptible to intravenous infection with hemolytic staphylococci. This is shown not only by the higher mortality rate of the mice but also by the higher microbial populations in the kidneys. Dubos and Schaedler(2) have found the same phenomenon even though approached from an entirely different aspect. The only 2 things in common between their experiments and those described herein are the reduction in intestinal flora and the use of the Giorgio strain of hemolytic *Staphylococcus aureus.* Nevertheless, the results are strikingly similar.

The mechanism of this unexpected result must be either with the host defenses or with the parasite. Dubos and Schaedler(2) used *Klebsiella pneumoniae* as well as staphylococci as the challenge infection and found the same general pattern of increased susceptibility in the "clean" animals. It seems reasonable to conclude, therefore, that reduction (by use of drug) or absence (by careful raising of the mice) of Gram negative enteric organisms has a deleterious effect on the animals' capacity to withstand microbial challenge. Considered in its broadest sense, the unaltered microbial flora of the intestinal tract of an animal are a type of host defense mechanism. The manner in which this is mediated is not at all clear. It certainly is alien to present concepts to associate a beneficial action with bacteria in relationship to the host. Except for the elaboration of Vit. K in the intestine there is no other evidence that the host is in any way helped by presence of these organisms.

It has been shown in this experimental model that penicillin has a beneficial effect on survival of mice infected with a penicillin resistant organism. The mechanism of this is discussed in detail by McCune(3,4) *et al.* In brief, studies of the Giorgio strain on agar plates with varying amounts of penicillin have revealed that this strain is composed of cells 90% of which are susceptible to 0.1 unit of penicillin per milliliter. However, even when organisms from one of the most resistant colonies are subcultured on plain agar or on penicillin containing agar the pattern of mixed resistance and susceptibility seen with the original culture is seen again. This would explain why penicillin is at least partially effective in treatment of this infection in mice.

Biology—One hundred seventy-nine Marks

Summary. Survival and microbial population studies in mice with their intestinal flora reduced by neomycin indicate that these animals are much more susceptible to intravenous staphylococcal infections than their littermate controls.

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Received May 25, 1960. P.S.E.B.M., 1960, v104.

Evaluation of Renal Function with Radio-Renogram.* (25979)

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Evaluation of renal function with I^{131} diodrast renograms has been reported(1,2). The value of the test is limited because the renogram could appear normal in presense of considerable kidney damage. This report deals with a modification of the I^{131} diodrast renograms designed to improve its effectiveness in both diagnosing and localizing renal disease.

Methods. Control Group. Standard I^{131} diodrast renograms were performed in 7 normal female mongrel dogs weighing between 10 and 17 kg. The animals were placed in a prone position and a catheter was inserted in the bladder. Scintillation detectors were placed over each kidney and over the heart. Output of the scintillation detector was connected to a count rate meter and a rectilinear strip chart recorder. Fifteen μc of I^{131} labeled diodrast (less than 3 mg) was injected intravenously. Levels of activity observed over each kidney and the heart were recorded. After completing the control renogram, an intravenous priming dose of para-amino-hippurate (PAH) calculated transiently to saturate the renal tubular mechanism was injected. The I^{131} diodrast renograms were repeated at frequent intervals as plasma level of PAH fell. Plasma levels of PAH were determined at each of these intervals. *Operative Group.* Routine radio-renograms were recorded in 8 dogs following which partial resection of their left kidneys were performed. Five dogs had about one-third, 2 had one-half and one had two-thirds of the left kidney excised. A bladder extrophy was also performed to facilitate study of indirect renal function in each kidney. One month or more post-operatively the glomerular filtration rate (GFR) and effective renal plasma flow (ERPF) were determined simultaneously in both kidneys of each dog by measurement of creatinine and para-amino-hippurate (PAH)

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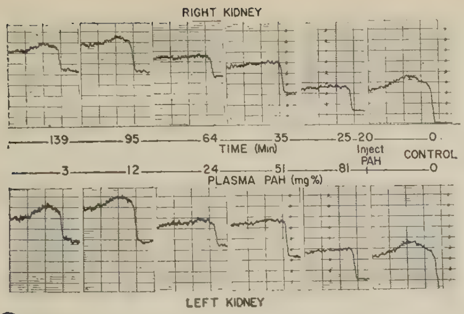
* Supported in part by research grants from Nat. Heart Inst., N.I.H., Bethesda, Md., and from Duke Univ. Research Council, Durham, N. C.



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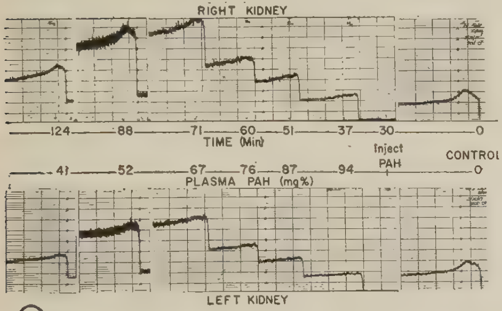

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FIG. 1. Radio-renograms pre- and post-intrav. inj. of 15 cc 20% PAH in normal 10 kg female dog.

FIG. 2. Radio-renograms pre- and post-intrav. inj. of 32 cc 20% PAH in a 14 kg female dog with ⅓ of left kidney removed.

clearances respectively(3). Routine radio-renograms were again recorded in each animal and in addition the tubular saturation technic with PAH was performed as described above.

Results. Control Group. Fig. 1. is an experiment representative of this series. The pre-PAH injection I^{131} renogram revealed normal concentration and secretion curves usually obtained. Following injection of the tubular saturation PAH dose the radio-renogram revealed no apparent concentration above initial blood dilution of I^{131} diodrast. Not until 95 minutes after the PAH dose when plasma PAH level was 12 mg % did the repeat renogram show some concentration and secretion of I^{131} diodrast. By 119 minutes when PAH plasma level was 3 mg % the I^{131} diodrast renogram had returned to essentially the control pattern.

Operative Group. Simultaneous recording of GFR and ERPF in both kidneys was measured in 3 animals with approximately one-third of the left kidney excised. GFR and ERPF of the left kidney averaged 56% and 55% respectively of normal right kidney. In 2 animals with one-half of left kidney removed a GFR and ERPF of 44% and 40% respectively of the normal right kidney was noted. When one-half or less of the left kidney was removed the I^{131} diodrast renograms gave inconsistent results, either showing no difference between the 2 kidneys or minimal differences.

After the saturation dose of PAH was injected, the immediate I^{131} renogram revealed essentially no concentration above initial blood dilution of the I^{131} diodrast and therefore no significant excretion of the substance. Between 3 and 50 minutes the normal right kidney was noted to show some concentration and excretion. The left kidney, depending on how much functioning tissue remained, showed either a delay in concentration and excretion or so minimal a concentration and excretion at any given moment as to be easily differentiated from the normal kidney. A representative case is noted in Fig. 2. Although GFR and ERPF of the left kidney were 65% and 58%, respectively, of normal values for the right kidney, no significant difference was noted in the control I^{131} diodrast renogram. At 37 minutes (or 7 minutes after tubular saturation dose of PAH) with a plasma PAH level of 94 mg % neither kidney showed any concentration of I^{131} diodrast. At 71 minutes (or 41 minutes after injection) at a PAH plasma level of 67 mg % the normal right kidney was able to concentrate and excrete the I^{131} diodrast whereas no significant similar function was seen in the left kidney. This difference was also noted at the next I^{131} diodrast injection at 88 minutes when plasma PAH level was 52 mg %.

Discussion. At low blood levels (less than 2 mg %) about 80% diodrast or PAH is taken up and secreted by the tubular cells in one circulation through the kidney. As plasma concentration of either substance is increased, more of it is secreted until maximal rate of secretion, Tm (tubular maximum) is reached. Further increases in concentration augment the rate of excretion only by increasing the filtered load which is that small fraction of circulating diodrast or PAH which is not protein bound(4).

Since concentration and secretion of I^{131} diodrast dose used in the standard test requires little kidney function, it is understandable why the renogram appears normal even when significant renal dysfunction exists. If the renal mechanism responsible for secreting diodrast is functioning at its maximum (for example, with Tm doses of diodrast or PAH), the I^{131} diodrast renogram would be expected to show a flat curve or essentially no tubular cell concentration or secretion of the I^{131} diodrast. Plasma level of the competing substance falls as the kidneys secrete it and/or it is distributed in extracellular fluids. Repeat I^{131} diodrast injections should show a gradual return of concentration and secretion by both kidneys. This sequence of events was demonstrated in normal dogs. If the functional capacity of one kidney is decreased the normal control kidney should show an earlier appearance of and a greater concentration and secretion of I^{131} diodrast as compared with the defective kidney. This was also demonstrated in the operative animals in this report.

Studies have been performed on human cases of unilateral and bilateral renal diseases. Currently we are employing a combination of I^{131} hippurate and PAH to eliminate the liver background problem encumbent with I^{131} diodrast(5).

Conclusions. (1) Routine I^{131} diodrast renograms frequently fail to demonstrate significant renal lesions. (2) Initial loading of tubular mechanism responsible for secretion of diodrast by competitive substance (for example, para-amino-hippurate) blocks concentration and secretion of I^{131} diodrast. (3) As tubules secrete the competing substance and plasma level gradually returns toward normal, the control kidney in cases of unilateral disease demonstrates, on successive injections of I^{131} diodrast, earlier appearance of uptake and secretion as well as greater quantitative uptake and secretion of I^{131} diodrast.

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Received June 8, 1960. P.S.E.B.M., 1960, v104.

Effects of Intra-Adrenal Infusion of Potassium on Urinary Potassium Excretion in the Dog.* (25980)

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It is well-known that intravenous infusion of potassium salts induces a prompt increase in urinary excretion rate of potassium. The kaliuresis is effected by a change in renal tubular activity since both glomerular filtration rate and serum potassium remain relatively constant(1). It is not known exactly how infusion of potassium salts provokes this change in renal tubular activity but it seems reasonable to suppose the existence of a site or sites sensitive to alterations in potassium concentration. Several lines of evidence suggest that at least one such site may be the adrenal cortex. There is a positive correlation between potassium intake and aldosterone excretion(2), and aldosterone acts directly on the mammalian renal tubule to increase potassium excretion(3). Furthermore, when potassium concentration is increased in perfusates of isolated beef adrenals, greater amounts of aldosterone are secreted(4). Similar results are obtained by addition of potassium to isolated rat adrenals(5). These observations suggest that potassium acts di-

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* This investigation was supported by Research Grants H-3166 and H-315 from Nat. Heart Inst., P.H.S., and was done during the tenure of a Research Fellowship of Am. Heart Assn. to the senior author.

rectly on the adrenal cortex to increase aldosterone secretion, aldosterone then inducing kaliuresis. If this interpretation is correct, infusion of a potassium-containing solution into the adrenal blood supply of an intact animal should have a quantitatively greater effect on potassium excretion than infusion of an identical solution into a non-adrenal artery. To test this hypothesis, the adrenal gland of the dog has been infused *in situ* with potassium in high concentration, the results being compared with those obtained when potassium was infused into a non-adrenal artery.

Materials and methods. Healthy mongrel dogs weighing 12 to 20 kg were maintained on a constant diet for at least 2 weeks before an experiment was performed. Food and water were withheld for 18 hours prior to an experiment. All animals were anesthetized with intravenous pentobarbital sodium. A solution containing, per liter, 145 meq of sodium, 5 meq of potassium, 110 meq of chloride, and 40 meq of bicarbonate was administered intravenously in every experiment by a constant-speed infusion pump at the rate of 1.0 ml/min. Urine was collected in graduated cylinders from either ureteral catheters or an indwelling bladder catheter. The femoral artery was cannulated with a polyethylene tube for blood sampling. After placing the animal on its right side, a left flank, subcostal incision and a muscle-splitting procedure were used to expose the retroperitoneal space. A small-bore polyethylene cannula, connected to a second constant-speed infusion pump, was inserted into the lumbar branch of the lumbo-adrenal artery and advanced proximally until its tip was just distal to the origin of the small fan-like adrenal branches. The cannula was anchored with a silk ligature placed around its tip. Infusate was thus carried to the adrenal gland with its normal arterial blood supply. The control series consisted of 5 dogs who underwent a surgical procedure identical with the one used for the experimental animals except that in these sham-operated controls, a non-adrenal artery was cannulated for potassium infusion. Infusion rate in both the adrenal and non-adrenal groups was 0.86 ml/min. After a suitable period of time, during which urine flow became relatively constant, 3 or 4 urine collection periods of 20 minutes each were obtained. The composition of the arterial infusate during these control periods was identical with that of the intravenous infusate. The arterial infusate was then changed to one differing from the control solution by addition of 45 meq of potassium chloride to each liter of solution. Urine collection periods of 20 minutes each were continued for 2 to 3 hours. At the end of each experiment, 1 ml of india ink was injected into the lumbar artery cannula. An experiment was considered technically successful if 75% or more of the adrenal cortex was densely stained with ink. A total of 6 experiments satisfied this criterion. Sodium and potassium were determined in serum and urine by indirect flame photometry, using lithium as an internal standard. The statistical methods used are those described in standard textbooks of statistics.

Results. Serum potassium. The mean serum potassium concentration for all the periods prior to potassium infusion was 3.81 meq/l in the control series and 3.92 meq/l in the adrenal infusion series. (Table I). Serum potassium increased slightly in 4 of the 5 control experiments and in 4 of the 6 adrenal infusion experiments. Mean increase during potassium infusion was 0.35 meq/l in control experiments and 0.17 meq/l in adrenal infusion experiments. The difference between the means for the 2 series could not be proved to be statistically significant (Table II).

Potassium excretion. Potassium excretion increased moderately in 4 of the 5 control experiments and in 5 of the 6 adrenal infusion experiments during infusion of potassium. Mean increase was 23 μeq/min in control series, and 16.3 μeq/min in adrenal infusion series. Although potassium excretion increased in both groups, the difference between the 2 groups could not be proved to be statistically significant.

K/Na ratio. There was no consistent change in urinary K/Na concentration ratio in either group during potassium infusion. The mean changes of 0.44 in control experiments and –0.24 in adrenal infusion experi-

TABLE I. Renal Effects of Systemic and Intra-adrenal Infusion of Potassium in Dog.

	Serum K (meq/l)			Urine K (μeq/min.)			Urine K/Na			Urine Na (μeq/min.)		
Dog No.	Control	Exp.	Δ	Control	Exp.	Δ	Control	Exp.	Δ	Control	Exp.	Δ
						Systemic infusion exp.						
1	3.73	4.40	+.67	55.6	82.7	+27.1	.50	.98	+ .48	110.4	84.6	−25.8
2	3.69	3.55	−.14	33.4	68.3	+34.9	1.66	.73	− .93	20.1	92.9	+72.8
3	3.67	4.29	+.62	22.4	49.3	+26.9	2.73	7.03	+4.30	8.2	7.0	− 1.2
4	4.07	4.35	+.28	44.1	36.9	− 7.2	.88	.94	+ .06	50.3	39.1	−11.2
5	3.87	4.22	+.35	22.6	55.9	+33.3	3.70	2.00	−1.70	6.1	27.9	+21.8
Mean	3.81	4.16	+.35	35.6	58.6	+23.0	1.89	2.34	+ .45	39.0	50.3	+11.3
						Adrenal infusion exp.						
6	3.96	4.07	+.11	55.8	52.9	− 2.9	.38	.47	+ .09	145.5	103.9	−41.6
7	4.37	4.31	−.06	59.1	83.8	+24.7	1.47	.79	− .68	40.1	107.4	+67.3
8	3.93	4.08	+.15	54.8	91.9	+37.1	4.00	1.79	−2.21	13.6	50.7	+37.1
9	3.80	3.99	+.19	58.5	73.0	+14.5	.95	1.63	+ .68	61.9	44.8	−17.1
10	3.83	3.79	−.04	40.5	52.6	+12.1	2.18	1.82	− .36	18.6	28.3	+ 9.7
11	3.61	4.29	+.68	14.1	26.2	+12.1	4.17	5.24	+1.07	3.3	5.0	+ 1.7
Mean	3.92	4.09	+.17	47.1	63.4	+16.3	2.19	1.96	+ .23	47.2	56.7	+ 9.5

ments could not be proved to be statistically significant.

Sodium excretion. There was no consistent change in sodium excretion in either group during infusion of potassium. The mean change was an increase of 11.5 μeq/min in control series and 9.5 μeq/min in adrenal infusion series. The change could not be proved to be statistically significant in either series.

Discussion. There can be little doubt that infusion of a solution containing 50 meq/l of potassium at a rate of 0.86 ml/min into an adrenal artery raises potassium concentration in the blood being supplied to that adrenal gland. This concentration is probably on the order of magnitude of 10 meq/l, as can be deduced from the following considerations. Rate of adrenal blood flow in the dog has been reported to be .297 ± .18 ml/kg/min (6). If there were a maximal flow of .5 ml/kg/min, a body weight of 20 kg, a hematocrit of 40% and a plasma potassium concentration of 3.8 meq/l, infusion of potassium in a concentration of 50 meq/l at the rate of .86 ml/min would be expected to elevate potassium concentration in adrenal artery plasma to 9.6 meq/l. This is a conservative estimate of the actual elevation of serum potassium concentration, higher concentrations probably being reached in the smaller animals. If the kaliuretic response to potassium infusion is mediated by the adrenal cortex, elevations in plasma potassium concentration of this magnitude should be a more than adequate stimulus for this response, provided they were not toxic to the gland. A toxic effect is unlikely as shown by the observation that potassium concentrations as high as 29.6 meq/l did not impair aldosterone production in isolated adrenal preparations(5). Since response to intra-adrenal infusion of potassium did not differ significantly from response to non-adrenal infusions, it can be concluded that the augmentation of potassium excretion is dependent upon some other mechanism.

TABLE II. Statistical Comparison of Renal Effects of Systemic *vs* Intra-adrenal Infusion of Potassium in Dog.

	Paired comparison of systemic *vs* adrenal series		
Parameter	Diff.	"t"	P
Serum K (meq/l)	.18	1.008	.3–.4
Urine K (μeq/min.)	6.73	.710	.4–.5
Urine K/Na	.68	.596	.5–.6
Urine Na (μeq/min.)	2.00	.086	>.9

These studies do not exclude the possibility that intra-adrenal infusion of potassium stimulates production of aldosterone, an effect which has been observed *in vitro*. On the other hand, in experiments on isolated calf adrenals(4) and isolated rat adrenals(5), the rise in aldosterone secretion evoked by high potassium concentrations was not quantitatively great. For this reason, Giroud and coworkers(5) suggested that alterations of serum sodium and potassium concentrations may modify aldosterone output by an indirect action through some other system. Regardless of the effect of serum potassium concentration on rate of secretion of aldosterone these experiments demonstrate that the characteristic kaliuresis accompanying potassium infusion is not dependent upon a potassium-sensitive receptor mechanism in the adrenal cortex.

Summary. 1) A technic has been described whereby the dog adrenal may be infused *in situ* without disturbing its arterial blood supply. 2) Utilizing this technic, infusion into left adrenal arteries of a solution containing high concentrations of potassium did not affect serum potassium concentration or urinary potassium excretion any differently than infusion of similar solution into a non-adrenal artery. It is concluded that the kaliuretic response to potassium infusions is not mediated by a direct action of potassium on the adrenal cortex.

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Received June 10, 1960. P.S.E.B.M., 1960, v104.

Electron and Fluorescence Microscopy of Mouse Hepatitis Virus.* (25981)

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Interest in comparative viral hepatitis stems from lack of methods for propagation and detection of the virus(es) of human hepatitis (1). Since identification of this agent depends on the hazardous exploitation of man, other host-specific viruses associated with hepatitis infections of dog(2), duck(3-5), and mouse (6-9) have been studied as possible prototypes. This communication describes procedures for visualization of the *Eperythrozoon*-free strain of mouse hepatitis virus (MHV 1)(10) by electron and by fluorescence microscopy.

Materials and methods. Newborn Swiss mice were infected *via* the peritoneal route with MHV 1(11). After 5 days, mice were sacrificed and infected livers were examined. Tissue imprints were prepared on coverslips, fixed without air drying in acid-alcohol, and stained with acridine orange fluorochrome at pH 3.8 and with fluorescein-tagged antibody by methods described for psittacosis infection of human cell lines(12). Antiserums were prepared in adult Swiss mice by repeated intraperitoneal inoculation of normal and of infective mouse-liver homogenates(13). Globulin fractions were conjugated with fluorescein isothiocyanate for use in the direct fluorescein-staining procedures(14). Paraffin sections were cleared in xylol, then stained with acridine orange and with fluorescein-tagged antibody. A Reichert Zetopan microscope and illumination system were used with appropriate ultraviolet light source and filters. Other paraffin sections and tissue imprints were stained by routine procedures with hematoxylin-eosin. Preparations of infected liver were fixed in Palade's(15) veronal acetate buffered osmium tetroxide, embedded in methacrylate, and sectioned for examination with an electron microscope (RCA EMU-3C).

Results. Tissue imprints and paraffin sec-

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* Supported in part by James W. McLaughlin Fellowship Fund and Dept. of HEW. The electron microscope was purchased and is maintained by a USPHS grant.

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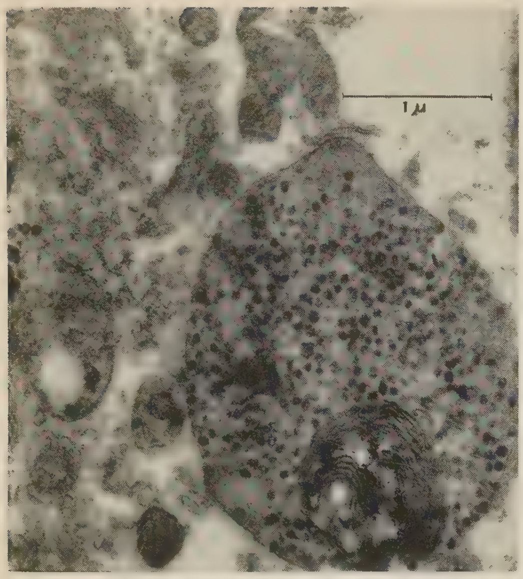

FIG. 1. Inclusion body in cytoplasm of hepatic cell infected with mouse hepatitis virus. Note limiting membrane and virus particles of varying densities. In some instances, dense-centered particles are surrounded by a single membrane. A portion of the nucleus is seen in upper-left corner.

tions which were stained with hematoxylin-eosin showed foci of necrosis in which pyknotic nuclei and cell fragments predominated. In comparison to the extensive liver damage, few cytoplasmic inclusions were observed. Although the dye binding nature of the inclusions could not be established in areas of cellular degeneration, eosinophilic bodies (1-5 μ) were present in otherwise normal appearing cells which surrounded necrotic areas. In parallel preparations stained with acridine orange, the cytoplasm of some hepatic cells contained abnormal accumulations of RNA-staining (red) material(16). Attempts to establish chemical identification of RNA-staining inclusions were made by treatment of infected tissue preparations with ribonuclease prior to staining with acridine orange. Ribonuclease removed RNA-staining (red) affinity of both cytoplasm and nucleoli and did not interfere with DNA-staining (yellow-green) affinity of the nucleoplasm for acridine orange. There was, however, some indication that ribonuclease did not change the RNA-staining property of a number of inclusions. With fluorescein-tagged antibody, positive-staining cytoplasmic inclusions were noted which corresponded to size, shape, and location of the RNA-staining inclusions. Observations by electron microscopy (Fig. 1) showed cytoplasmic inclusions (1-5 μ) which contained aggregates of virus particles (90 ± 20 mμ). Some inclusions were ruptured and elementary bodies were scattered throughout the cytoplasm. Other sections showed mature virus particles emerging from a viroplasm in which faint outlines of immature organisms could be seen.

Discussion. The cytological observations of Armstrong and Niven(16) on mouse livers infected with MHV 1 were confirmed. Thus, it was noted, also, that infected hepatic cells stained with acridine orange contained abnormal accumulations of material in the cytoplasm which fluoresced intensely red. Attempts to establish the chemical identity of these inclusions by treatment with ribonuclease prior to staining with acridine orange were not considered conclusive. Observations were hampered by 1) fragments of pyknotic nuclei, degenerated cells, and nuclear "blebs" which fluoresced both red and yellow-green; 2) the small size of the elementary body which precluded their detection unless larger aggregates were present; and 3) the possibility that if MHV 1 is an RNA virus, it would fluoresce the same color as the cytoplasm and hence be obscured. Although the nucleic acid composition of MHV 1 is still an open question, preparations stained with fluorescein-tagged antibody showed inclusions of similar size, shape, and location which were antigenic to homologous antiserum.

As determined by electron microscopy, intact cytoplasmic inclusions were surrounded by a limiting membrane(s). Virus particles within these inclusions had an average diameter of 90 ± 20 mμ (Fig. 1). This size range is comparable to that estimated by Gledhill *et al.*(17) who used filtration through membranes of graded porosity. Occasionally, the membrane around the inclusion was broken and single particles or small aggregates, some with limiting membranes, were noted throughout the cytoplasm. Presence of virus in similar liver samples was shown by infectivity tests in newborn mice.

Summary. Livers of mice infected with

mouse hepatitis virus (MHV 1) were examined by conventional staining procedures and by electron and fluorescence microscopy using acridine orange fluorochrome and fluorescein-tagged antibody. Preparations stained with acridine orange showed abnormal accumulations of RNA-staining material in cytoplasm of hepatic cells. Inclusion bodies of similar size, shape, and location were stained selectively with fluorescein-tagged antibody. Observations by electron microscopy showed virus particles in cytoplasm which measured 90 ± 20 mμ in diameter.

The authors are indebted to Dr. R. H. Rigdon and his staff for invaluable technical assistance.

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Received June 20, 1960. P.S.E.B.M., 1960, v104.

Influence of Age at Time of Irradiation on Induction of Leukemia and Ovarian Tumors in RF Mice. (25982)

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Among physiological variables influencing susceptibility to radiation-induced leukemia in mice is age at time of irradiation(1,2). In man also, according to epidemiological surveys, susceptibility to leukemia induction may be relatively high during embryonic and fetal development(3), and the hematologic type of leukemia induced may vary with age at the time of irradiation(4). The present investigation was carried out to extend earlier observations on the relation between age at irradiation and leukemogenesis in mice of the RF strain.

Methods. Male and female RF mice of several ages from 3 days before birth to 180 days after birth were exposed to whole-body X radiation in doses of 100, 200, and 300 r (Table I). Radiation was administered with a 250-kvp General Electric Maxitron machine at 30 ma, with 0.1 mm of inherent and 3 mm of added Al filtration (hvl, 0.44 mm of Cu), TSD 93.7 cm, and a rate of 75-100 r/min. Suckling young were exposed together in intact litters, whereas other mice were exposed individually by the technic described previously(5). The mice were necropsied on death and tissues fixed in Zenker-formol for sectioning.

Results. Incidence of granulocytic leukemia was not significantly increased by irradiation *in utero* or during the first 9 days after birth but was increased by irradiation later in life, susceptibility to induction of the disease rising gradually to a maximum at 70 days of

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* Operated by Union Carbide Corp. for U. S. Atomic Energy Comm.

TABLE I. Leukemia Incidence in Relation to Age at Irradiation.

Age at irradiation, days	X-ray dose, r	Mice		Mean after-survival time, mo	%		
		Sex	No.		Myeloid leukemia	Thymic lymphoma	Ovarian tumor
1	0	♂	368	19	3	5	
		♀	161	20	3	6	1
	100	♂	58	19	2	4	
		♀	55	18	2	18	44
	200	♂	67	17	8	9	
		♀	62	17	5	14	66
–1 to –3*	300	♂	21	18	0	0	
		♀	15	14	0	0	7
1–9	300	♂	124	12	5	15	
		♀	59	16	5	24	61
14	300	♂	101	14	26	13	
		♀	94	14	6	34	39
28	300	♂	80	12	42	14	
		♀	87	14	14	37	41
40	300	♂	104	10	43	19	
		♀	94	9	13	51	30
70	300	♂	65	12	54	8	
		♀	94	12	26	21	26
180	300	♂	111	10	32	9	

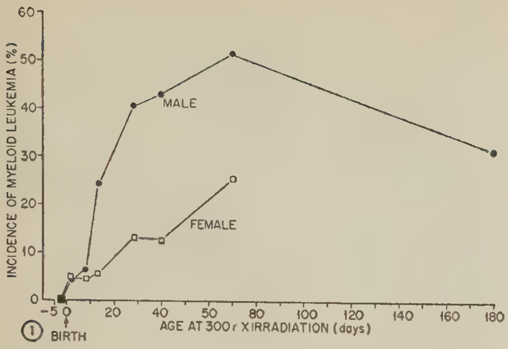
* Irradiated *in utero* 1-3 days before birth.

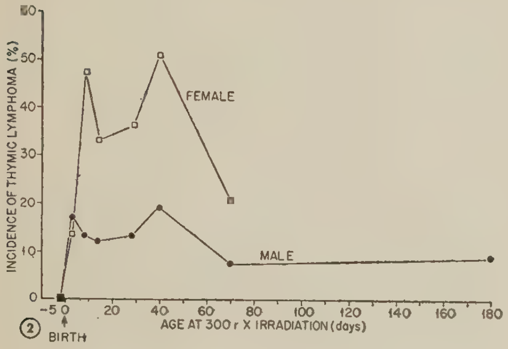
age (Table I, Fig. 1). Thymic lymphoma, likewise, was apparently not induced by irradiation *in utero* but was readily induced by irradiation immediately after birth (Fig. 2). Susceptibility to its induction decreased between 40 and 70 days of age coincidentally with spontaneous involution of the thymus (Fig. 3). The higher susceptibility of males to myeloid leukemia and of females to lymphoma agrees with earlier observations (2). Mortality during the first 30 days after irradiation was negligible in all age groups. Mean after-survival time was less consistently correlated with age at irradiation than with leukemia incidence but decreased with increasing radiation dose, as did mean induction time for leukemias. Ovarian tumors were significantly less common in the mice irradiated *in utero* than in those irradiated after birth (Table I).

Discussion. From earlier results(2), we expected susceptibility to induction of myeloid leukemia to be relatively low during the first few days of life, despite concomitantly high susceptibility to lymphoma induction. We did not expect, however, that susceptibility to both types of leukemia would be low in the fetus, especially in view of epidemiologic data suggesting a high susceptibility in the human fetus or infant(3). Although there was no evidence of radiation lethality in the exposed fetuses, as judged by number of viable offspring, it is conceivable that 300 r may have been too large a dose for maximum leukemogenesis at this age despite its leukemogenetic potency later in life. In irradiated adult RF mice, incidence of myeloid leukemia declines as dose is increased from 300 to 450 r(2). These experiments need to be extended, therefore, with smaller doses of radiation, exposures at other stages of gestation, and larger numbers of mice per group.

Since susceptibility to ovarian tumor induction was also relatively low in the mice irradiated *in utero* (Table I), radiosensitivity as such may vary appreciably with age of the host, for reasons yet to be elucidated. In this connection, it is noteworthy that sensitivity of the mouse ovary to sterilization by radiation is relatively low during the period shortly before birth(6, W. L. Russell, personal communication). In addition, of course, age-dependent variations in the $LD_{50/30}$ of radiation for mice are well documented(7).

Another factor of theoretical importance in susceptibility to leukemia development is the





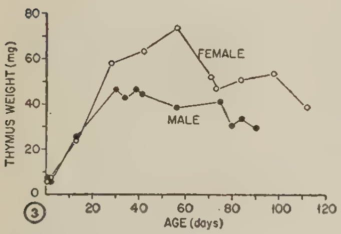


FIG. 1. Incidence of myeloid leukemia in relation to age at irradiation.

FIG. 2. Incidence of thymic lymphoma in relation to age at irradiation.

FIG. 3. Thymic wt in relation to age.

rate of turnover of cells in leukocyte-forming tissues, since selection and proliferation of leukemic variants are presumably favored under conditions of high mitotic activity. Consistent with this idea is the observation that susceptibility to induction of thymic lymphomas tends to decline with natural or hormone-induced thymic involution and to increase with thymic hyperplasia(8). Although rate of cell division in the fetal thymus is not known, irradiation *in utero* causes less depression of thymic weight than irradiation after birth (A.C. Upton and E. P. Sniffen, unpublished data). As regards myelopoietic activity, the number of myeloblasts in the body increases progressively with body weight throughout the first weeks of life (A. C. Upton and E. P. Sniffen, unpublished data). Thus, if the probability of myeloid leukemia induction varies with number of myeloblasts irradiated, susceptibility to radiation-induced leukemia should increase with age in the period of growth and development, as has been observed. That this correlation is not entirely consistent, however, is indicated by the rather sudden increase in susceptibility to myeloid leukemia induction between the first and second weeks of life (Table I), and by the disproportionate degree of protection against leukemia induction afforded by shielding a small amount of hemopoietic tissue(2).

An additional factor to be considered is the possible role of viruses in pathogenesis of radiation-induced leukemia(9-12). If leukemia is assumed to result from activation of a leukemogenic virus present at time of irradiation, age-dependent epidemiologic variations in exposure to the virus might account for the observed variations in susceptibility, as might passive immunization by maternal antibodies or the presence of other viruses that inhibit or enhance the leukemia agent.

Summary. RF mice exposed to 100-300 r of whole-body X-rays at various ages from before birth to 180 days after birth showed age-dependent variations in susceptibility to leukemia induction. Susceptibility to induction of granulocytic leukemia was minimal when irradiation was carried out during gestation or shortly after birth, rising later to a maximum at about 70 days of age. Susceptibility to induction of thymic lymphomas was, likewise, apparently minimal during gestation but maximal shortly after birth, declining later in life at the time of thymic involution. Susceptibility to induction of ovarian tumors was relatively low in mice irradiated *in utero*.

We are grateful to F. F. Wolff, E. S. Ledford, and W. D. Gude for technical assistance and to Dr. A. W. Kimball for statistical analysis of data.

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Received June 23, 1960. P.S.E.B.M., 1960, v104.

Survival of Mice in Absence of Inert Gas.* (25983)

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It has been generally assumed, for lack of evidence, that the nitrogen gas of the earth's atmosphere plays no role as a respired gas in the metabolic function of higher animals. However, since N_2 at pressures above 2 atmospheres begins to interfere with the normal behavior of the nervous system(1), it appears justified to ask whether or not it may at .79 atmosphere exert some effect on animal systems. The answer to this question is to be sought by looking for any biological changes in animals maintained in absence of N_2 gas. As a means of freeing the environment of N_2 gas, substitution of another inert gas for N_2 is not satisfactory since the other gas may of itself modify the results. On the other hand an atmosphere of pure O_2 at normal pressure is unsuitable since this will prove lethal in a matter of a few days. To test the possible long-term effects of absence of any inert gas upon the function of animals it is therefore necessary to use an atmosphere of pure oxygen but at reduced pressure to avoid oxygen toxicity. Our experiments indicate that mice will survive for long periods of time in oxygen when its pressure is maintained at 197 mm Hg. This is the level which provides the same inspired O_2 tension (150 mm Hg at BTPS) as breathing air at 1 atmosphere. Furthermore, 197 mm Hg of pure O_2 is the atmosphere which provides for a satellite cabin in space the lowest pressure differential across the walls compatible with a normal inspired O_2 tension within.

Method. The major problem is a mechanical one, that of maintaining animals for long periods of time in an atmosphere of O_2 at a reduced pressure. To avoid introduction of N_2 gas from the air into the chamber, the arrangements must render access to the animals unnecessary during the course of an experiment lasting many weeks. Chamber pressure and oxygen ventilation must be automatically maintained within narrow limits by reliable means and temperature and humidity must be kept within the comfortable range. In addition food and water must be provided and excreta conveniently accommodated. Since it took many months of trial and error to arrive at a satisfactory solution, a brief description of our arrangement follows. With the exception of the watering device, the gas sampling system and the water vapor trap,† all other parts are commercially available. The apparatus is schematically represented in Fig. 1. A small cast-aluminum chamber‡ (10

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* This investigation was supported in part by Wright Air Development Command, Wright-Patterson Air Force Base, Ohio.

† An acrylic resin chamber sealed around the cooling coil of a small vapor absorption type refrigeration unit (Astral Refrigerette).

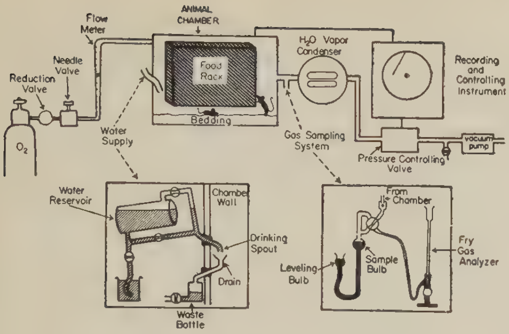

FIG. 1. Altitude chamber system for long-term maintenance of mice in oxygen at reduced pressure. Chamber is continuously flushed with O_2, flow regulated by needle valve. Controlling valve in outflow line is operated by recording pressure controller to maintain chamber pressure constant. Water supply and gas sampling systems shown in detail.

x 10 x 18″ inside) with a windowed door (7 x 12″) housed the animals. The chamber was continuously flushed with a stream of "medical" O_2. A reducing valve was followed by a needle valve across which the gas pressure dropped from above to below atmospheric pressure. Oxygen flow into the chamber was controlled by the needle valve and continuously monitored by a rotameter. To maintain CO_2 tension in the chamber below 5 mm Hg the required flow was calculated from the alveolar ventilation equation, putting in, for "CO_2 production," that of all the mice in the chamber, and for "alveolar ventilation," the oxygen flow. In both chamber and alveoli the steady-state gas concentrations are independent of volume of the ventilated space. Transcribing the alveolar ventilation equation (2) to the ambient temperature and pressure conditions of our experiment, one obtains this relation: chamber CO_2 (mm Hg) is equal to .83 x CO_2 output of all mice (ml, STPD, per min.) divided by oxygen flow (1, ATPD, per min.). Thus, for example, if CO_2 output of 6 mice is equal to 10 ml/min., then a flow of 2 liters/min. of O_2 at the reduced pressure will maintain (10 x .83)/2 or 4 mm P_{CO_2} in the chamber. Periodic analysis of the chamber gas confirmed these predictions. CO_2 concentration was never observed to exceed 5 mm P_{CO_2} and was usually lower. Chamber gas samples were obtained periodically by the device shown in Fig. 1. A small sample of gas was aspirated from a T tube in the exhaust line by means of a mercury reservoir. This gas could then be compressed after turning a stopcock and shunted directly through a connecting tube into a Fry gas analyzer for CO_2, O_2 and N_2 analysis. This procedure not only allowed one to monitor CO_2 concentrations but also to detect any inward leaks of N_2 which might occur during the weeks of uninterrupted experiments. The inflowing gas was continuously removed from the chamber by a vacuum pump after first passing over a refrigerated vapor trap to remove the moisture. The pressure was maintained by a servo-operated pressure controlling valve§ and was continuously recorded throughout the whole experiment. Thus any failure to maintain pressure could be easily detected. The sensitivity of the pressure controlling valve was ± 2 mm Hg. The temperature of the chamber was maintained between 24° and 27° by a thermostat operated heater. The mice were bedded on 2″ of Sterolit.‖ This powdery material absorbed all the waste products and prevented their further decomposition. Food was provided in the form of Purina Fox Chow or Old Guilford mouse pellets. These were placed in a closed wire mesh container which held enough food for duration of experiment. Water was supplied from the outside by means of a system illustrated in Fig. 1. This system was found to be necessary since simpler devices failed to work adequately under reduced pressure. By proper manipulation of stopcocks additional water could easily be supplied at any time. At start of experiment the chamber was flushed with 100% O_2 which the animals breathed for about 10 minutes. The chamber was then evacuated until final pressure was reached in about 15 minutes. All animals used were Swiss mice with the exception of four C57

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‡ The authors are greatly indebted to the Dept. of Physiology, School of Aviation Medicine, Randolph Air Force Base, for the loan of this chamber.

§ Motosteel Evenaction valve, actuated by a recording Fulscope absolute pressure controller (Taylor Instrument Co., Rochester, N. Y.).

‖ "Sterolit", Minerals & Chemicals Corp. of America, Menlo Park, N. J.

TABLE I. Accident-Free Sojourns of Mice in 197 mm Pure O_2 Atmosphere.

Exp. #	Mice		Days in chamber
1	6	adult male	55
3	6	2-mo "	18
8	6	adult female	12
	11	2-14 day fetus	"
9	2	lactating female	19
	18	suckling young	"
10	2	adult female	51
	6	fetus—young (conception to 27 day)	48
	1	adult male	22
	2	" female	"
	14	fetus—young (conception to 1 day)	"
	4	C 57 black female	"
	4	C 57 Swiss fetus	5

black females used in the breeding experiment.

Results. In 5 different experiments (summarized in Table I) mice were maintained in pure O_2 at 197 mm Hg total pressure for a total of 1308 mouse-days (53 different mice) with no apparent ill effects. In several other experiments a particular type of lethal accident occurred. This will be described below.

Young mice passed all the stages from conception to birth to weaning and adolescence in the chamber in an apparently normal fashion. Microscopic examination of 14-day fetuses, and of the eyes of those conceived and born in the chamber, revealed no abnormalities.

Of the adult mice that were kept in the chamber one group of 6 males after 55 days appeared normal as revealed by histological sections of heart, kidney, spleen, liver, lungs, thyroid, and eyes. Other groups remained apparently healthy and carried on the functions of mating, fertilization and implantation of ova, gestation, bearing and suckling of young in a normal fashion. The longest uninterrupted stay in the chamber was 51 days, by 2 females which conceived, bore and raised young there.

In the breeding experiment one male put into the chamber with 8 females (4 Swiss, 4 C57 black) impregnated the 4 Swiss females within the first 3 days and at least 2 of the C57 females about 2 weeks later. The 4 Swiss females bore their young in the chamber. The first 2 litters were removed from the chamber, along with the mothers, one day after birth. All 14 of these young removed from the chamber survived and developed normally for at least 8 weeks. The second 2 litters were left undisturbed in the chamber, where the 2 mothers ate all but 6 of the young within the first day or two. The surviving 6 were then raised to 27 days of age in the chamber before ever being exposed to normal air. The retinas of young conceived and born in the chamber and of the 2 females which stayed there 51 days were later given careful histological examination, which showed them to be apparently normal.

The one adverse effect observed in these experiments was the death of some mice within the first 48 hours of exposure to the reduced pressure. This phenomenon appeared to be a result of the decompression itself, since it never occurred after the first 48 hours. In a total of 115 mouse decompressions 20 mice died. Typically the mice that died this way became relatively inactive immediately after the reduction in pressure, and appeared to sleep most of the time until death. In the late stage they tended to sit hunched up, with fur erect, breathing in large gasps. On autopsy their lungs were found to be almost completely atelectasized. Of particular interest were four young mice which died. These animals were conceived, born and raised in the chamber in the absence of inert gas. They were finally brought out into normal air at 27 days of age. After 2 hours of breathing air for the first time in their lives they were again placed in the chamber and returned to their original environment of O_2. They succumbed within 24 hours.

Discussion. The only statement that can be made on the basis of these observations is that life and reproduction are possible for a mammal in the absence of all but a small fraction of the inert gas that is present in air. This life appears, on gross observation at least, to be essentially normal with the exception that fatalities may occur immediately following decompression.

It seems likely that these fatalities are due to collapse of alveoli following minute obstructions (mucous) of the smallest airway. Recent evidence(3,4) suggests the existence of a normal tendency of some alveoli to collapse, a tendency that is ordinarily kept in check by reexpansion of collapsed alveoli upon deep inspiration (sigh). It has been shown (5) that under 1 atmosphere of pressure rate of collapse following obstruction is 60 times faster on pure O_2 breathing than on air breathing. When pure O_2 is breathed the collapse of a blocked-off alveolus is determined only by removal of O_2 by the perfusing blood. (CO_2 removal is secondary to O_2 removal, which by raising concentration of CO_2 in the remaining gas, reverses the normal CO_2 gradient.) Thus the time for collapse, assuming perfusion rate to be constant, should be approximately proportional to number of molecules of O_2 present. Therefore, the effect of lowering total pressure at which pure O_2 is breathed will, by lowering absolute volume-concentration of O_2, speed up the collapse of obstructed alveoli, in accordance with the following relation:

$$\text{Collapse time} = k\,P_{A_{O_2}} = k\left(P_B - P_{A_{H_2O}} - P_{A_{CO_2}}\right).$$

In the case of $P_B = 197$ mm Hg, therefore, $P_{A_{H_2O}}$ and $P_{A_{CO_2}}$ being assumed to remain normal at 47 and 40 mm Hg respectively, the collapse time should be approximately (197-87)/(760-87) or 1/6 of that for $P_B = 1$ atmosphere. This then would suggest that under our experimental conditions rate of collapse of a blocked alveolus is increased about 360 times above the value for breathing air at 1 atmosphere. It is conceivable that at this rate the tendency to collapse may in some circumstances prove too much for the forces which normally operate to restore the alveoli. However, this account does not explain why the deaths observed should all occur during the first 48 hours in the chamber. It may be speculated that all the mice that died were suffering from some condition in which increased bronchiolar secretions rendered them more prone to airway obstruction and atelectasis.

Conclusion. It is concluded that an inert gas such as N_2 is not necessary for maintenance of normal metabolic processes. However, the proneness for development of pulmonary atelectasis is exaggerated in absence of an inert gas such as N_2.

Summary. 1) Mice were maintained for 51 days in an atmosphere of oxygen at total pressure of 197 mm Hg. This provides a normal inspired O_2 tension. CO_2 and N_2 pressures in the ambient atmosphere did not exceed 5 mm Hg for either gas. 2) Under these conditions animals usually did not encounter any difficulties. Several litters were born and in one case young were conceived, born and raised in virtual absence of inert gas. However, in several cases animals died within 48 hours after being subjected to this environment. Once past this period only mechanical failure of the apparatus terminated these experiments. It is suggested that pulmonary atelectasis was responsible for these early deaths.

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Received June 24, 1960. P.S.E.B.M., 1960, v104.

Distribution and Excretion of Radioactivity in Rats After Oral Administration of H^3-3-Methylcholanthrene.[*] (25984)

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3-methylcholanthrene, a carcinogenic hydrocarbon of extremely high potency, will induce mammary cancer in susceptible strains of mice and rats when administered by mouth. Dose-response relationships, optimal conditions for tumor induction, and influence of hormones on tumor growth have been described for the rat by Huggins, *et al.*(1). According to these investigators, a single 10 mg dose induces mammary cancer in 20% of female Sprague-Dawley rats, age 50 days. The studies presented here were undertaken to acquire additional information regarding absorption, tissue localization (with particular reference to the mammary gland), and excretion of a carcinogenic dose of this hydrocarbon labeled with tritium.

Methods. Female Sprague-Dawley rats, age 50 days, were used. In the first of two experiments, concentration of tritium was determined in selected tissues, urine and feces of 3 rats 24 hours after administration of 10 mg H^3-3-methylcholanthrene by gastric tube. In the second experiment, daily excretion of tritium in urine and feces of each of 4 rats given a single 10 mg dose of H^3-3-methylcholanthrene by gastric tube was determined. H^3-3-methylcholanthrene, tritiated by the Wilzbach procedure(2)[†] and recrystallized to constant specific activity (2,000,000 dpm/mg disintegrations per minute) was dissolved in sesame oil to make a final concentration of 10 mg/ml. After administration of 1 ml of this solution, followed by 1 ml of sesame oil through a No. 8 French catheter, each animal was placed in an all-glass metabolism cage designed for complete and separate collection of urine and feces. On termination of the experiment, tissues for analysis were removed, weighed and minced with a fine scissors. Three 50-100 mg samples of each tissue were taken for analysis of tritium by the combustion method of Jacobson, *et al.*(3). The remainder of the sample was homogenized in distilled water and extracted 4 times with toluene. A 5 ml aliquot of the extract was added to 10 ml of "fluor"[‡] solution and counted in a Packard Tricarb liquid scintillation spectrometer. This technic will recover 85% to 95% of known quantities of 3-methylcholanthrene added to tissue homogenates. The residue was dried *in vacuo* and analyzed by the combustion technic. Feces were dried in air, then ground to a fine powder in a mortar. Three 20 to 40 mg samples from each 24 hour collection were taken for analysis by the combustion technic. Each 24 hour urine sample was analyzed in triplicate by directly counting 0.3 ml in 4.8 ml of absolute ethanol and 10 ml of "fluor" solution. The efficiency of the counting system was determined by means of an internal standard, and all results were expressed as dpm.

TABLE I. Percentage Recovery of Tritium in Female Sprague-Dawley Rats 24 Hr after Administration of a Single Dose of 10 mg H^3-3-Methylcholanthrene by Gastric Tube.

Rat	1	2	3
Absorbed	6.5	5.6	5.3
Urine	4.9	4.4	3.9
Tissues	1.6	1.2	1.4
Unabsorbed	62.4	57.9	93.1
Stomach and contents	6.3	.2	31.9
Intestines and contents	32.1	37.0	52.8
Feces	24.0	20.7	8.4
Total	68.9	63.5	98.4

Results. Exp. 1. The percentage recovery of tritium 24 hours after administration of 10 mg H^3-3-methylcholanthrene is presented in Table I. Percentage of radioactivity recovered in tissues, urine and feces by the combustion technic was approximately 69 for rat

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[*] This investigation was supported by Jane Coffin Childs Memorial Fund for Medical Research, Am. Cancer Soc., and U.S.P.H.S.

[†] Dr. H. I. Jacobson and Dr. T. N. Jacobsen of Ben May Laboratory for Cancer Research prepared the tritium-labeled 3-methylcholanthrene used in these experiments.

[‡] The "fluor" solution is prepared by dissolving 7.5 g diphenylaxazole, 0.075 g "POPOP" and 120 g naphthalene in 500 ml spectroscopic grade dioxane and 500 ml of redistilled xylene.

TABLE II. Total Radioactivity (dpm/g Wet Wt) in Tissues 24 Hr after Administration of a Single Dose of 10 mg H^3-3-Methylcholanthrene by Gastric Tube (Combustion Technic).

Rat No.	1	2	3	Mean ± S.E.
	× 1000			
Kidney	32.9	56	56.3	48.4 ± 7.71
Fat	31.6	24.6	20	25.4 ± 3.37
Liver	23.9	24.6	23.7	24.1 ± .28
Mammary gland	11.1	23.4	19.1	17.97 ± 2.20
Lung		15.1	9.1	12.1 ± .44
Uterus	11.3	7.1	17.4	11.9 ± 2.99
Brain		4.9	4.1	4.5
Skeletal muscle	2.2	2.5	3.3	2.6 ± .32
Blood	2.5			

#1, 64 for rat #2, and 98 for rat #3. Fifty-eight to 93% of the administered dose was recovered in gastric and intestinal contents and feces. It is assumed that these values represent, for the most part, material which has not been absorbed. Approximately 6% (6.5, 5.6 and 5.3, respectively) of administered radioactivity was found in the tissues analyzed, or in urine. Values obtained for urine (4.9, 4.4 and 3.9%, respectively) for the 3 animals were in good agreement.

Less than 2% of administered radioactivity was recovered from the tissues analyzed. The concentration per gram wet weight is presented in Table II for the combustion technic and in Table III for the toluene-extraction method. The results of the combustion method, which determines total radioactivity, were higher than those obtained by toluene extraction. This is to be expected inasmuch as the aqueous phase of the toluene-water system was not analyzed. The results presented in Table III represent only the concentrations found in the toluene extract and those in the dried residue.

With the exception of brain and skeletal muscle, all tissues analyzed contained at least 7000 dpm/g wet weight. Average concentration of total radioactivity ranged from 11,000 dpm/g for uterus to 48,000 for kidney. Mammary tissue and fat contained nearly comparable levels of total radioactivity; average concentrations were 18,000 and 25,000 dpm/g, respectively.

Sixty to 70% of the radioactive material in fat and breast tissue was toluene-soluble; less than 10% was extracted from the other tissues analyzed. These results suggest (1) that the radioactive material is in 2 (or more) chemical combinations, *i.e.*, one that is readily soluble in toluene and another which is insoluble, or (2) that the material is less firmly bound to adipose tissue.

Exp. 2. The daily excretion of tritium in urine and feces of 4 rats following a single feeding of 10 mg of H^3-3-methylcholanthrene is presented in Fig. 1 for urine and Table III for feces. Radioactivity was detected in urine of Rat No. 1 for 13 days, Rat No. 2 for 17 days, Rat No. 3 for 16 days, and Rat No. 4 for 15 days. With the exception of Rat No. 2 whose peak urinary excretion occurred on the second day, 3.5 to 4.0% of administered dose was excreted within the first 24 hours. These results are in agreement with those presented for Exp. 1. Detectable quantities (5 dpm/mg) of radioactive material appeared in feces of Rat No. 1 for 11 days, Rat No. 2 for 8 days, Rat No. 3 for 9 days and Rat No. 4 for 7 days. Of the original radioactivity administered, 71% for rat No. 1, 59% for Rat No. 2, 52% for Rat No. 3, and 45% for Rat No. 4 was recovered in feces during the first 5 days; trace amounts occurred intermittently throughout the remainder of observa-

TABLE III. Radioactivity in Toluene-Soluble Fraction and in Residue after Toluene Extraction, of Tissues from Sprague-Dawley Rats 24 Hours after Administration of 10 mg H^3-3-Methylcholanthrene by Gastric Tube.

	dpm/g wet wt					
	Rat 1		Rat 2		Rat 3	
	Extract	Residue	Extract	Residue	Extract	Residue
	× 1000					
Kidney	1.72	22.5	1.8	20.4	1.73	
Fat	20	.68	19.6	.48		
Liver	1.85	16.5	2.5	23	1.85	15.8
Mammary	11.5	2.77	13.5	.59	10.2	1
Lung			.66	9.5	.69	3.04
Brain			.4	2.4	.2	1.94
Skeletal muscle	.3	.824	.4	.63	.23	.9

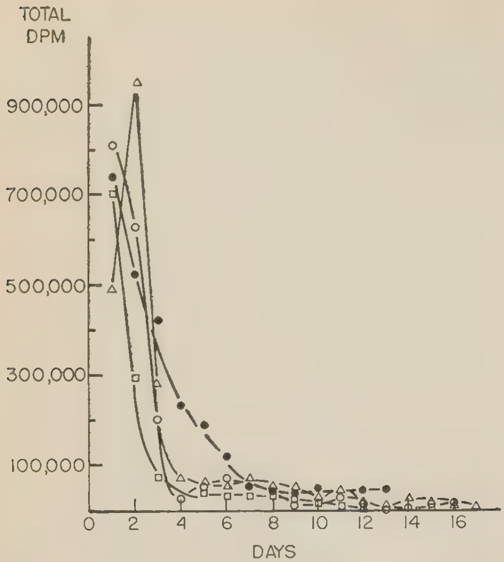

FIG. 1. Daily excretion of tritium in urine of 4 rats after administration of a single 10-mg dose of H^3-3-methylcholanthrene by gastric tube. 2000 dpm is equivalent to 1 μg of original hydrocarbon.

tion period (25 days). Trace quantities of radioactive material were found in mammary gland and liver but not in the other tissues from Rat No. 1 at 26 days.

Discussion. It is estimated that less than 10% of administered radioactivity was absorbed. The data obtained by toluene extraction are in good agreement with those of Dao, *et al.*(4), who analyzed a benzene extract of hydrolyzed tissue spectrofluorophotometrically. If the toluene-extractable radioactivity obtained in this study proves to be 3-methylcholanthrene, and on this point there is no information, concentration of the carcinogen per gram of tissue for the 3 rats is estimated to be 5.8, 6.8 and 5.1 μg for mammary gland, and 10 and 9.8 μg for fat, respectively. It would appear that this low concentration is sufficient to induce carcinogenesis in highly susceptible animals. This estimate is in agreement with observations in this laboratory that a single subcutaneous injection of 10 μg of 3-methylcholanthrene will induce sarcoma at site of injection in 2 of 10 rats.

Relatively high levels of tritium were recovered in all tissues except skeletal muscle and brain. Concentrations in kidney and liver were higher than those of mammary gland and fat. In contrast to fat and breast tissue, approximately 50% of total radioac-

TABLE IV. Excretion of Tritium in Feces after Administration of a Single Dose of 10 mg of H^3-3-Methylcholanthrene by Gastric Tube (Combustion Method), on 25 Successive Days.

Rat 1		Rat 2		Rat 3		Rat 4	
Total dpm (× 1000)	dpm/mg	Total dpm (× 1000)	dpm/mg	Total dpm (× 1000)	dpm/mg	Total dpm (× 1000)	dpm/mg
128	162	1,530	1,190	2,233	1,722	1,650	1,320
12,730	3,700	1,905.25	11,040	4,330	14,340	5,314	8,100
1,100	336	7,450	11,900	2,794	10,310	1,837	1,780
145	165	2,252.2	1,995	610.24	4,420		
102	147	648.8	406	477.32	2,010	312	172
15.4	32	93.23	81	1,635	694	86.6	36
21.13	21	230.55	56	897.6	548	12.81	8
5.44	16	9.914	6	191.17	122	2.4	1
3.04	8	4.57	1	51.82	20	.832	.3
12.04	7	3.14	1	4.85	2	2.2	1
6.5	5	1.38	1	.94	1	.0	0
2.65	3	2.99	2	2.46	1	1.54	1
.37	3	2.2	2	.505	2	.745	1
6.09	5	4.16	1	2.04	1	.177	1
3.08	3	1.1	1	.0	0	.24	.2
5.03	3	2.2	2	1.82	1	1.37	1
.42	1	2.59	1	3.36	1	1.415	1
1.69	2	1.53	1	.733	1	.68	1
4.89	1	1.15	1	6.4	3	6.36	5
3.64	2	1.3	2	.83	1	4.47	4
11	4	8.33	4	2.4	1	6.8	4
7.34	5	4.33	1	4.15	3	.99	1
16.6	6	1.9	1	.36	.3	2.77	1
8.25	4	2.364	2	.6	.3	1.67	1
11.48	9	.68	.4	.58	.3		

tivity was not extracted by water or toluene, but remained in the dried residue fraction. These results suggest that the administered hydrocarbon is rapidly converted to radioactive metabolites that are firmly bound to tissue. This type of protein binding has been established with other carcinogenic agents (5,6). Studies to determine the chemical nature of the binding of H^3-3-methylcholanthrene or its metabolites with tissue constituents are being undertaken.

The prolonged excretion of radioactive material in feces indicates that the carcinogen or its metabolites have a relatively long sojourn in the organism. The observations of Dao, *et al.*(4) and of Dauben and Mabee(7) support this interpretation. Failure to demonstrate significant quantities of radioactivity in tissues 25 days after administration of the hydrocarbon does not preclude its presence in trace amounts. It must be emphasized that the present technics for determination of tritium in biological material are not sensitive enough to detect with certainty concentrations below 5 dpm/mg. The small and variable quantities found in the feces after the first week may be due in part to the limitations inherent in the technic and in part to biological variation.

Summary. 1) Sprague-Dawley female rats, age 50 days, were given 10 mg H^3-3-methylcholanthrene dissolved in sesame oil by gastric tube. 2) In the first experiment selected tissues, urine and feces were analyzed for radioactivity after 24 hours. Sixty to 90% of administered radioactivity was found in gastric and intestinal contents or in feces; 4.4% in urine. Less than 2% was recovered in the tissues analyzed. Results obtained by combustion technic for measurement of total radioactivity were compared with those obtained by toluene extraction. Data indicate that most radioactivity in fat and mammary tissue could be extracted with toluene, whereas negligible quantities could be obtained by this method for liver, kidney, brain, uterus and skeletal muscle. 3) In the second experiment, 24-hour samples of urine and feces were analyzed for total radioactivity for 24-25 days. Significant quantities of tritium-labeled material appeared in urine for 13-17 days and in feces for 7-11 days. Thereafter, fecal and urinary excretion of radioactivity occurred intermittently in trace amounts.

We wish to thank Miss Beverly Cockrell for technical assistance.

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Received June 24, 1960. P.S.E.B.M., 1960, v104.

Action of Desoxycorticosterone Acetate* on Glycogen. I. Relationship of Liver Glycogen Formation to Potassium Metabolism. (25985)

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It has been established that a relationship exists between potassium and carbohydrate metabolism in the intact animal. Harrop and Benedict(1) were first to describe a decrease in blood serum potassium concentration following administration of insulin. They believed that this decrease was the result of potassium entering cells of tissue along with glucose during glycogenesis. This idea was supported by Fenn(2) who observed that potassium content of liver of intact rats approximately paralleled glycogen content. Gardner (3) reported that rats depleted of potassium by dietary restriction of this element produced practically no glycogen following intraperitoneal injection of glucose. Desoxycorticosterone acetate (DOCA) has been shown to be a potent mineralocorticoid. When injected into experimental animals, it caused a wasting of potassium resulting in a severe deficiency of that element(4). Low tissue glycogen levels have been reported(5) following administration of large doses of this steroid. *In vitro* studies showed that glycogen formation in liver slices was dependent on a high potassium concentration in the incubating medium (6). Similar studies indicated that even in the presence of high potassium concentration, addition of 1.0 mg % DOCA to the medium completely inhibited glycogen production(7). It was of interest therefore, to study the mechanism of action of DOCA on glycogen formation *in vivo,* and to determine whether the inhibition elicited by this steroid on glycogen production was secondary to, or independent of its action on potassium metabolism. It was postulated that by increasing potassium intake of rats, large decreases in potassium content of tissues and blood serum, normally associated with administration of large doses of DOCA, might be avoided. The action of DOCA on glycogen formation could thus be studied independently of large changes in potassium concentration of both serum and tissues.

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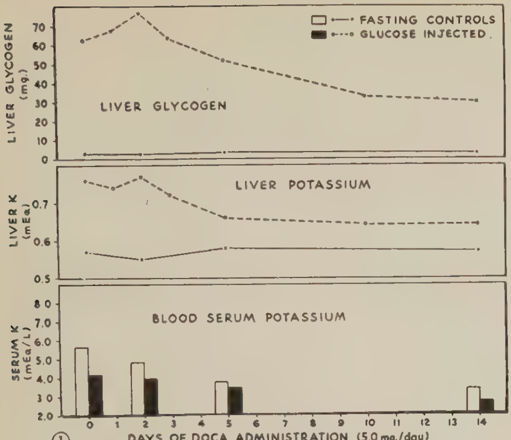
* Generously supplied by Organon, Orange, N. J.

Materials and methods. Two hundred and twenty male rats of Wistar origin that weighed between 200 and 300 g were divided into 2 groups. Group I consisted of 140 rats which were further divided into 7 subgroups of 20 rats each. Half of the rats in each subgroup were given tap water to drink and the other half were given 1.0% potassium chloride solution. Rats in the first subgroup received daily injections of sesame oil and served as controls for the other 6 subgroups that received 5.0 mg of DOCA daily in the form of a 5.0% solution in sesame oil, injected subcutaneously for 1, 2, 3, 5, 10 and 14 days respectively. Twenty-four hours before last injection, food was withdrawn from the cages but the drinking fluid continued to be available *ad libitum.* At time of last injection, 200 mg of glucose per 100 g of body weight were injected intraperitoneally in the form of a 10% solution prepared in isotonic saline. After 4 hours the animals were anesthetized by injecting 5.0 mg of sodium pentobarbital intraperitoneally per 100 g of body weight. Fifteen minutes later the abdominal cavity was opened and blood was collected from the inferior vena cava for potassium analysis. The liver was removed, quickly trimmed of fat and connective tissue, and excess blood removed by blotting between sheets of filter paper. Approximately half of each lobe was placed in a tared weighing bottle containing 10 ml of 5.0% trichloroacetic acid and finely minced with scissors for glycogen determination. The remaining portion of liver was placed in a small tared silica dish and ashed for potassium analysis. Glycogen was determined by the method of Carroll *et al.*(8) which consisted of trichloroacetic acid extraction of the homogenized tissue, and alcohol precipitation of the glycogen, followed by the anthrone reaction. Potassium was determined with a Beckman Model DU Flame Spectrophotometer(9) following the analytical procedure of Bills *et al.*(10). The results from the analytical determinations were averaged for each subgroup of animals. Mean liver glycogen and potassium values were expressed in milligrams per liver and meq per liver respectively. Mean blood serum potassium values were expressed in meq/l. Group II consisted of 80 rats subdivided into 4 subgroups of 20 rats each. Rats in the first subgroup received daily injections of sesame oil and served as controls for the other 3 subgroups that received 5.0 mg of DOCA per day for 2, 5 and 14 days respectively. Half of the animals in each subgroup were given tap water to drink and the other half 1.0% potassium chloride solution. These rats served as fasting-controls for the glucose injected rats of Group I, and were treated identically except that 0.9% sodium chloride solution was substituted for the 10% glucose solution injected intraperitoneally 4 hours before sacrifice.

Results. The results obtained with animals that received tap water are presented in Fig. 1. In the fasting control rats, administration



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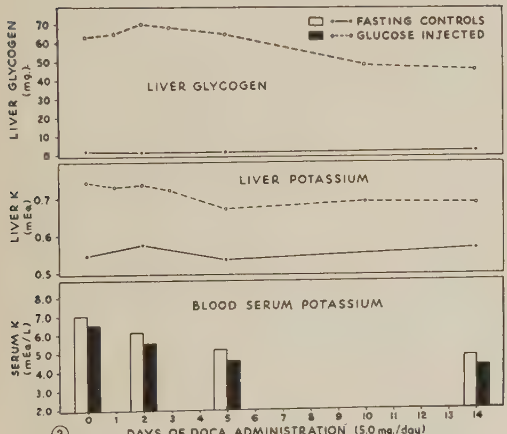

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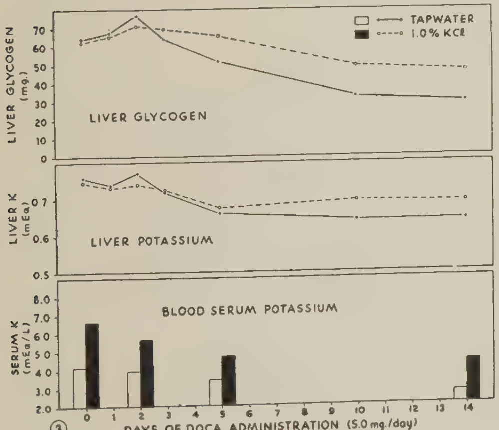

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FIG. 1. Effect of 11-desoxycorticosterone acetate (DOCA) on glycogen and potassium content of liver 4 hr after the I.P. inj. of glucose into intact rats.

FIG. 2. Effect of 11-desoxycorticosterone acetate (DOCA) on glycogen and potassium content of liver 4 hr after the I.P. inj. of glucose into intact rats drinking 1.0% KCl solution.

FIG. 3. Effect of 11-desoxycorticosterone acetate (DOCA) on glycogen and potassium content of liver 4 hr after the I.P. inj. of glucose into intact rats drinking 1.0% KCl solution compared with those drinking tapwater.

of DOCA had essentially no effect on either mean glycogen or mean potassium content of the liver. There was however, a marked decrease in blood serum potassium concentration from a mean of 5.67 meq/l for the control group to a mean of 3.31 meq/l for the group of rats treated with 5.0 mg of DOCA for 14 days.

Following intraperitoneal injection of glucose, mean glycogen and mean potassium contents of liver from the rats that did not receive DOCA increased 59.1 mg and 0.187 meq respectively. Glycogen production was facilitated by administration of DOCA for either one or 2 days, but was inhibited in the rats that received the steroid for 5 days or more. Inhibition of glycogen formation was approximately paralleled by a decrease in potassium content of both liver and blood serum. Statistical studies showed that the decreases in both potassium and glycogen content were significant in the rats that received DOCA for 5 days or more.

The results obtained with animals that received 1.0% potassium chloride solution were presented in Fig. 2. As in the studies with fasting rats that received tap water, DOCA had essentially no effect on either glycogen or potassium content of liver. However, there was a decrease in blood serum potassium concentration of 2.09 meq/l for the rats that received DOCA for 14 days.

In rats that received DOCA for less than 3 days, the glycogenic response and increase in liver potassium content following glucose administration was essentially the same as that observed in those that received tap water as shown in Fig. 3. For the glucose injected rats treated with DOCA for 5 days or more, those that received potassium chloride solution had a higher blood serum potassium concentration and produced more glycogen than those that received tap water. These differences were statistically significant.

Discussion. Conflicting reports have appeared concerning the action of DOCA on glycogen formation in intact animals. Koch and Bohlander(11) found that administration of 1.0 mg DOCA per day for 6 days to intact rats resulted in a one-third increase in liver glycogen concentration. Winter and Thompson(12) reported that implantation of 5 mg pellets of DOCA subcutaneously in intact rats was without effect on glycogen concentration. Verzar and Wang(5) showed that administration of a single 20 mg injection of DOCA intraperitoneally completely inhibited glycogen formation in the liver of intact rats. These conflicting reports can be explained in part on the basis of the results of the present studies. These studies showed that DOCA administered daily for short periods of time resulted in increased liver glycogen production, but when administered daily for longer periods of time, there was a decrease in glycogen production.

The observation that potassium entered the liver during glycogenesis was in agreement with the report by Fenn(2). Since decrease in glycogen formation approximately paralleled decrease in potassium content of liver and blood serum, these data suggested that DOCA induced inhibition of glycogen formation was secondary to potassium depletion. The observation that glycogen formation was essentially normal in DOCA treated rats that had an increased intake of potassium chloride further supported this mechanism of action of DOCA on glycogen formation. These data also suggested that it was the availability of potassium from extracellular fluid that limited production of glycogen in the livers of DOCA treated rats rather than potassium content of the liver.

Summary. 1. Daily administration of DOCA for short periods caused an increased glycogenic response in liver of rats following intraperitoneal injection of glucose. Daily administration of DOCA for longer periods resulted in decreased glycogenic response. 2. DOCA induced inhibition of glycogen formation was accompanied by decrease in potassium content of both liver and blood serum. 3. By forcing an increase in potassium intake, the potassium content of both liver and blood serum was maintained at essentially normal levels and liver glycogen production was not significantly inhibited in rats that received DOCA. 4. These data indicated that DOCA induced inhibition of glycogen formation was secondary to potassium depletion.

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Received June 27, 1960. P.S.E.B.M., 1960, v104.

Effect of Arginine Deficiency on Nutritional Muscular Dystrophy in the Chick.* (25986)

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Nutritional muscular dystrophy in the chick is of complex dietary etiology. The disease, in chicks, is affected by dietary amounts of Vit. E, methionine, cystine, and selenium. Addition to the deficient diet of either Vit. E or one of the sulfur-containing amino acids completely prevents the condition(1,2). Supplementing the basal diet with selenium results in partial prevention of muscular dystrophy (3,4). During investigations as to whether the effects of the sulfur amino acids were specific for preventing the disease, deficiencies of other essential amino acids were studied to determine effect on development of muscular dystrophy. A study of the effect of an arginine deficiency disclosed an interesting interaction between dietary arginine level and development of muscular dystrophy. The following experiments show that with an arginine deficiency nutritional muscular dystrophy did not develop in the chicks, even though the diet was also deficient in Vit. E and the sulfur-containing amino acids.

Experimental. Male and female White Plymouth Rock chicks hatched from eggs produced by a flock of hens maintained on a Vit. E-low diet were used in all experiments. They were housed in thermostatically-controlled batteries with wire mesh floors. The basal diet used was as follows: casein, 20.00; cerelose, 66.56; cellulose, 3.00; stripped lard, 4.00; mineral mixture, 5.49; vitamin mixture, 0.95. The vitamin mixture has been described by Morrison *et al.*(5) and contains no Vit. E. The mineral mixture supplied the following in g/kg of diet: $CaHPO_4$, 21.51; $CaCO_3$, 14.92; KH_2PO_4, 8.67; NaCl, 6.00; $MgCO_3$, 1.42; $FePO_4 \cdot 4H_2O$, 2.65; $MnCl_2 \cdot 4H_2O$, 0.463; KI, 0.0026; $Cu(C_2H_5O_2)_2$, 0.0119; $ZnCl_2$, 0.1; $CoCl_2 \cdot 6H_2O$, 0.001; $NaMoO_4 \cdot 2H_2O$, 0.0083; KCl, 1.5.

Muscular dystrophy was readily diagnosed by appearance of grossly visible white striations running in the direction of muscle fibers on the breast of the chicken, as described by Dam *et al.*(1). The chicks were sacrificed at the end of each experiment and the skin removed from the breast to make the final assessment of incidence and severity of muscular dystrophy.

Experiment 1 was designed to study effects of deficiencies of arginine, glycine and methionine on muscular dystrophy. These amino acids were added to the basal diet alone, and in all possible combinations. The effects of Vit. E were also studied with the basal diet and in a diet with arginine and glycine added. The levels of amino acids used were chosen because these were the levels present in a previous basal diet(3) containing casein and gelatin as protein sources, which had been the standard diet for study of muscular dystrophy in this laboratory. The results of this experiment are included in Table I. Muscular dystrophy was observed only in the chicks receiving arginine without added Vit. E or methionine. No muscular dystrophy was observed in the chicks receiving the basal diet or this diet supplemented with glycine.

Since growth of the chicks receiving the arginine deficient diets was considerably less than in chicks receiving diets adequate in arginine, a second experiment was conducted in which one group of chicks receiving the basal diet plus arginine was pair-fed with the basal group to eliminate the difference in size of the chicks receiving these 2 diets. In other treatments, chicks receiving additions of arginine alone, arginine plus methionine, and arginine plus Vit. E were fed these diets *ad libitum.* The results of this experiment (Table II) confirm those of the previous experiment in that supplemental arginine was found to be essential for development of muscular dystrophy even when the growth of those receiving added arginine was restricted by limited feed intake.

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* This work was aided by grants from Muscular Dystrophy Assn. of America, Inc., Nutrition Foundation, Inc., and Distillation Products Industries, who also supplied the stripped lard and *d*-α-tocopheryl acetate used in the studies.

TABLE I. Amino Acid Deficiencies of Casein and Muscular Dystrophy in the Chick.

Treatment	Avg wt, 5 wk, g	Muscular dystrophy
Basal	209 (16)*	0/16†
+ .4% DL-methionine	172 (7)	0/17
+ 1.01% L-arginine HCl	378 (4)	11/14
+ 2.74% glycine	262 (17)	0/17
+ .4% methionine + 1.01% L-arginine HCl	455 (14)	0/14
+ .4% DL-methionine + 2.74% glycine	218 (17)	0/17
+ 1.01% L-arginine HCl + 2.74% glycine	416 (16)	12/16
+ *idem* + .4% DL-methionine	419 (8)	0/ 8
+ 80 mg/kg d-α-tocopheryl acetate	206 (16)	0/16
+ 1.01% L-arginine HCl + 2.74% glycine + 80 mg/kg d-α-tocopheryl acetate	376 (15)	0/15

* No. of survivors.

† Expressed as No. of chicks showing symptoms (numerator) over No. of chicks examined (denominator).

Vit. E or methionine supplementation prevented development of muscular dystrophy.

A third experiment was conducted to determine whether higher levels of arginine would increase the severity of the disease in chicks. The basal diet was supplemented with levels of 1.01, 2.08 and 4.15% arginine HCl. The arginine-supplemented groups were all fed the same amount of feed as the basal group after 5 days of age. Results of this experiment (Table III) confirmed the previous 2 experiments in that supplemental arginine was necessary for development of muscular dystrophy in young chicks. Severity of the muscular dystrophy was measured by means of an index. Affected muscles were given a score of from 1 to 10 depending on amount of breast muscle affected by the lesions. A score of 10 indicated the most extensive muscular degeneration. Based on this scoring system, higher levels of arginine had no significant effect on the severity of the dystrophy.

TABLE II. Effect of Arginine on Development of Muscular Dystrophy.

Treatment	Avg wt, 4 wk, g	Muscular dystrophy	Feed/chick
Basal	216 (17)*	0/17§	420
+ 1.01% arginine HCl†	248 (18)	17/18	392
+ *Idem*‡	298 (19)	18/19	489
+ 1.01% arginine HCl + .4% DL-methionine	380 (20)	0/20	565
+ 1.01% L-arginine HCl + 80 mg/kg d-α-tocopheryl acetate	304 (19)	0/19	510

* No. of survivors.

† Fed same amount of feed as basal after one wk of age.

‡ *Ad libitum* fed.

§ See footnote †, Table I.

To obtain further evidence on a possible relationship between arginine level, growth and muscular dystrophy, another experiment was conducted in which several groups of chicks were given the arginine-deficient diet for 5 weeks, whereupon some of the groups were then switched to the diet with adequate arginine supplementation. Within 2 weeks thereafter (when the chicks were 7 weeks of age) all chicks receiving arginine showed muscular dystrophy while those not receiving arginine still did not appear to be affected by the disease. Feed intake was equalized in this experiment again to eliminate large differences in body size.

TABLE III. Effect of Various Levels of Arginine on Development of Muscular Dystrophy.

Treatment	Avg wt, 5 wk, g	Muscular dystrophy	Severity index
Basal	204	0/20*	—
+ 1.01% L-arginine HCl	261	17/20	7.0
+ 2.08%	270	19/19	8.4
+ 4.15%	261	18/20	8.0

* See footnote †, Table I.

Discussion. These experiments indicate another dietary variable of importance in nutritional muscular dystrophy in the chick. Development of nutritional muscular dystrophy in chicks appears to depend not only on levels of Vit. E and sulfur-amino acids, but also upon level of arginine in the diet.

The specific reason for the relationship between arginine deficiency and muscular dystrophy observed in this study is not known. Differences in rate of growth did not appear to be important, since muscular dystrophy was equally severe when growth of the chicks given adequate arginine was limited by restricted feeding.

A possible explanation of the results obtained in these experiments may lie in the effect of the sulfur-amino acids on muscular dystrophy. Since arginine was the first limiting amino acid in the basal diet, utilization of the remaining amino acids of the diet for protein synthesis was depressed. More methionine and cystine would then become available to the animal for use in other functions, one of which may be involved in prevention of muscular dystrophy. If this is the case, deficiencies of essential amino acids other than arginine may have the same dystrophy-depressing effects.

These experiments also provide evidence that the sulfur amino acid effect is not a nonspecific effect, observed only because of a deficiency of an essential amino acid. As opposed to the effect of methionine a deficiency of the essential amino acid arginine did not result in muscular dystrophy in chicks fed a Vit. E deficient diet.

Summary. Nutritional muscular dystrophy in the chick did not occur when an arginine deficient diet was fed even though the diet was low in Vit. E and sulfur amino acids. When arginine was included in the basal diet, a high incidence of muscular dystrophy was observed by 5 weeks of age, even when growth was restricted in the chicks receiving adequate arginine by paired feeding with the basal group.

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Received June 29, 1960. P.S.E.B.M., 1960, v104.

Growth of Human Cancer Cells (HEP 2) in Newborn Rats.* (25987)

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Transplantation of antigenically foreign tissue is usually followed by a prompt host reaction resulting in rejection of the transplant unless special conditions prevail. Conditions which permit growth of heterologous tissues include special implantation sites such as the brain and the anterior chamber of the eye(1,2), "conditioning" of recipient animal by agents such as cortisone and x-ray which cause a general depression of defense mechanisms(3), and use of an embryonic or newborn recipient such as the chick embryo(4). Each of these methods has been used successfully for the heterologous growth of human cancer cells. More recently it has been reported that the human epidermoid cancer cell line HEp #3 will grow in rats inoculated *in utero* (5) in newborn unconditioned hamsters[†], and in newborn unconditioned mice(6). The present report describes growth of another human epidermoid carcinoma cell line (HEp #2) in normal newborn rats.

Methods. The HEp #2 cell line of Toolan (7) was grown in monolayer tissue cultures, harvested with trypsin and washed once with Gey's salt solution. The cells were resuspended in Gey's solution, counted in a hemocytometer, and adjusted to 8 to 10 million cells per ml. Non-inbred albino rats less than 24 hours old were inoculated intravenously in the facial or submental veins with 0.25 ml of this suspension. Inoculation failures were discarded from this study. Rats which died within 2 to 3 days after inoculation were also excluded. Litter mate controls were inoculated with an equal volume of Gey's salt solution.

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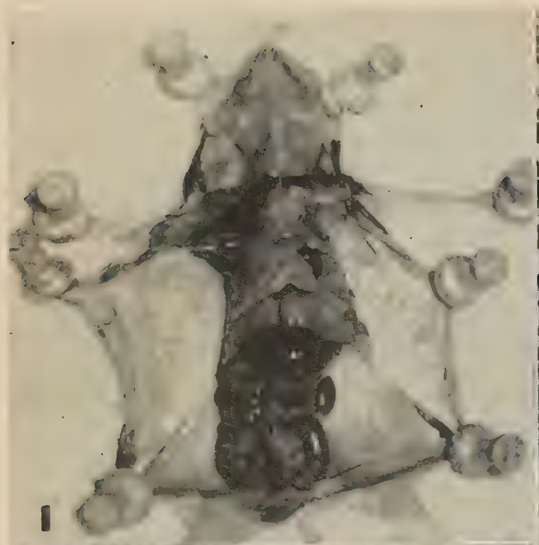
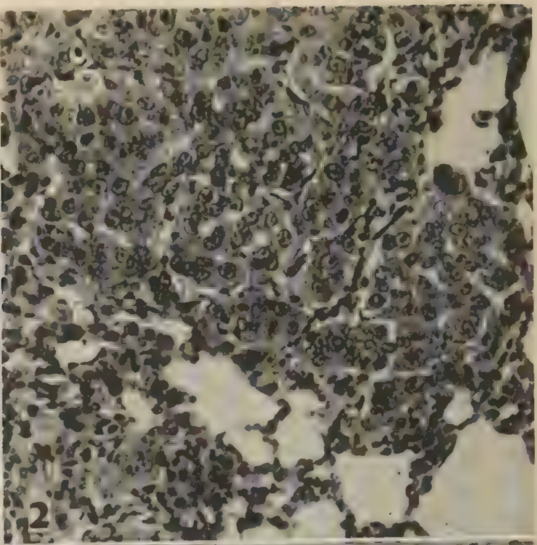
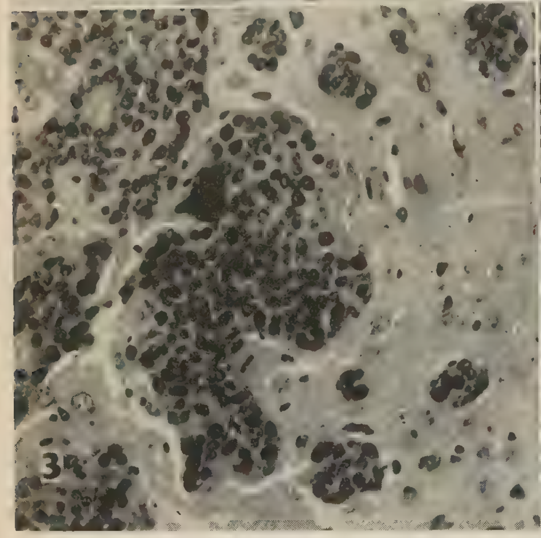
* This study supported by research grants from Nat. Cancer Inst., U.S.P.H.S., and Phoebe Waterman Fund.

[†] Personal communication.







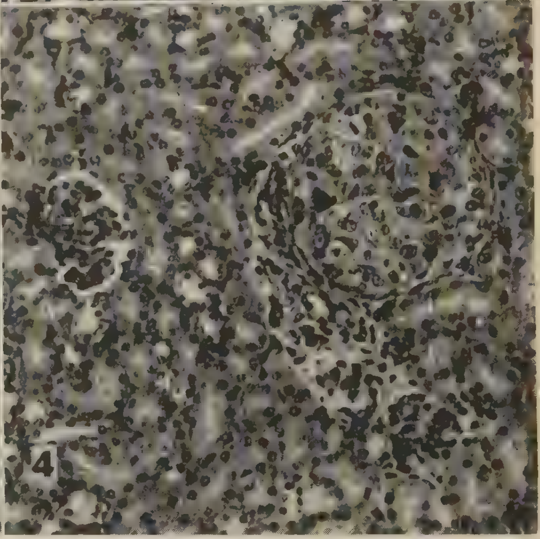


FIG. 1. Massive tumor in lung of rat 26 days old inoculated with HEp 2 cells intrav. when approximately 4 hr old.

FIG. 2. Histologic section of lung of rat age 26 days inoculated with HEp 2 cells intrav. when approximately 10 hr old. Lung parenchyma collapsed and displaced by epidermoid cancer cells.

FIG. 3. Multiple foci of tumor cells in brain stem of rat 27 days old inoculated with HEp 2 cells intrav. when 6 hr old.

FIG. 4. Histologic section of kidney of rat 26 days old inoculated with HEp 2 when approximately 10 hr old. Bowman's capsule is filled with tumor cells which displace glomerular tuft.

Results. As early as 15th to 19th day cell inoculated rats showed signs of illness. By 4 to 5 weeks their weight was 30% to 50% less than that of litter-mate controls. Terminally, they had ruffled fur, severe cachexia, thoracic enlargement, rapid and labored breathing, cyanosis, and sometimes signs of central nervous system disorder. Death usually occurred in the 4th or 5th week. At the end of 5 weeks 92% (77/84) had died of respiratory failure or had been sacrificed when moribund. Mortality in litter-mate controls was only 3% (1/35).

All animals were autopsied except a few which were cannibalized or severely autolyzed. On gross examination the lungs of all cell inoculated rats were enlarged, firm, white, and did not collapse when the thorax was opened (Fig. 1). Other organs rarely had gross abnormalities.

Microscopic examination consistently revealed masses of the HEp #2 cancer cells almost completely obliterating normal lung tissues (Fig. 2). Cancer cells were also found in brain (Fig. 3), and kidney (Fig. 4). The frequency with which lesions occurred in organs other than lungs is not known because these lesions were not visible grossly and only lung was sectioned routinely.

Subcutaneous and intraperitoneal inoculations of newborn rats and intravenous, intraperitoneal or subcutaneous inoculations of newborn mice with 100,000 to 1,000,000 cells gave no evidence of transplant growth or stunting of the recipients.

These studies are being continued to determine effect of age of rats and size of inoculum on growth of the implant, and to study other human cell lines. These incomplete studies suggest that progressive growth of HEp #2 will occur only with an inoculum in the magnitude of a million or more cells and in rats less than 3 days of age. The cell lines HEp #1, J-111 and Detroit 6 grow in a similar manner.

Discussion. The factors permitting growth of these heterotransplants are unknown and are being investigated further. The simplest explanation is that the human cells were introduced at an age when rejection mechanisms were incompetent, and grew at the expense of lung tissue to a size where death was caused by anoxia before rejection could be initiated. Alternative possibilities are that early introduction of these heterologous cells caused specific immune tolerance or immunologic "paralysis" due to antigen excess.

The human identity of this HEp #2 cell line was established by antigenic studies with the tanned erythrocyte agglutination technic during these experiments.

Summary. Tissue cultured cells of human cancer cell line HEp #2 inoculated intravenously into newborn rats at a dose of 1 million or more cells resulted in growth of the heterologous tumor cells in lungs and sometimes in other organs and produced death by respiratory impairment in 4 to 5 weeks.

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Received June 28, 1960. P.S.E.B.M., 1960, v104.

Diurnal Rhythm in Plasma Corticosterone and Lack of Diurnal Rhythm in Plasma Compound F-like Material in the Rat.* (25988)

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A diurnal variation in activity of the adrenal cortex has been shown to occur in the mouse, dog and human being(1-5) on the basis of changes in circulating levels of eosinophils and in concentrations of the adrenocorticoids in blood and urine. In conjunction with a study on corticoids of rats, it was deemed advisable to examine the possibility of such a diurnal variation in plasma adrenocorticoid concentrations. Recently, Guillemin *et al.*(6)

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* Aided in part by grant from Purdue Research Foundation.

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demonstrated a diurnal variation in levels of corticosterone in the blood of the rat and cited the importance of such variations in determination of resting steroid levels. Present results confirm the above observation but, in addition, indicate a lack of diurnal rhythm in another material of adrenal origin found in the plasma.

While a few paper chromatographic analyses of rat plasma have indicated the presence of more than a trace of 17-hydroxycorticosterone (Compound F), several workers have reported the presence of a Porter-Silber chromogen in plasma extracts(7,8). Since Silber and Porter reported that corticosterone (Compound B) reacted with their color reagent and had an absorption maximum near 350 mμ(9), a way was open to determine the compound in plasma. If indeed, 2 steroids were present in rat plasma, with different absorption maxima, technics of simultaneous determination could be applied(10,11). While this possibility was being investigated, Rosenman *et al.* reported on such a simultaneous determination of corticoids applied to rat plasma(12).

During this study, evidence was found for the presence of an unknown material in rat plasma, reacting with the Porter-Silber reagent and having an absorption maximum near 410 mμ(13). Additional data indicated the component to be of adrenal origin. Since colorimetric studies on 17-hydroxycorticoids in plasma have involved a chromogenic comparison of plasma extracts with Compound F, in this study the absorption of the unknown material was also compared with that of Compound F. For this reason the unknown substance is termed "F-like material" (to indicate color comparison to the standard) which should not be taken to infer identification as a 17-hydroxycorticoid.

Methods. Groups of 5-10 rats of the Purdue strain, 60 to 90 days of age and all of the same sex, were placed in a large wire cage in a light proof room. The room was illuminated with artificial light for 12 hours each day, 8 a.m. to 8 p.m., and was maintained under controlled temperature conditions. The animals were fed the standard laboratory diet and given tap water *ad libitum.* The animals were allowed to acclimate to the new environment for 14 days before plasma samples were obtained for analysis.

Four time intervals were selected as the periods during which blood samples would be obtained: 9-10 a.m., 2-3 p.m., 9-10 p.m., and 2-3 a.m. At the beginning of one of the intervals, the animals were given a single intraperitoneal injection of Nembutal (Abbott) 30 mg/kg body weight, and were removed from the room 20 minutes later when all were anesthetized. Blood was withdrawn by cardiac puncture using a heparinized syringe and plasma obtained by centrifugation. Plasma from 3 to 5 animals was pooled to obtain a 15 ml sample which was stored in the deep freeze for subsequent corticoid determination.

The technic for extraction was that of Peterson *et al.*(14) modified for use with rat plasma(13). In all cases, plasma samples were extracted with dichloromethane and the extract divided into two 10 ml aliquots. One aliquot was treated with the Porter-Silber reagent, phenylhydrazine, in a sulfuric acid-alcohol mixture, and the other aliquot was treated with the alcohol-sulfuric acid mixture as a blank. Absorbancy values for plasma extracts and corticoid solutions of known concentration were obtained at 355 and 410 mμ. In all cases, absorbancy values of the chromogen were corrected for background by subtracting the acid blank absorbance. These corrected absorbancy values were used in simultaneous equations to calculate levels of corticosterone and the unknown substance in rat plasma (F-like material).

When this technic was applied to plasma of completely adrenalectomized rats, absorbancy values of the chromogen obtained at both 355 and 410 mμ, when corrected for background color by subtracting the acid blank, were zero. These data indicated that both corticosterone and the F-like material were absent from the blood of adrenalectomized rats. With rare exception, corrected absorbancy values at both wave lengths in samples from normal rats were greater than zero.

Results. Levels of the corticoids in the peripheral blood of the rat were found to vary as a function of time of day (Table I). In both male and female rats, highest level of corticosterone was found at the 9-10 p.m.

TABLE I. Peripheral Plasma Corticoid Levels in Normal Rats at Various Time Intervals.

Time	No. of groups	μg % Comp'd B	μg % Comp'd F-like
		Male	
9-10 a.m.	3	11.8	11.3
2- 3 p.m.	7	22.0	12.7
9-10 p.m.	6	31.2	9.3
2- 3 a.m.	3	7.2	11.1
		Female	
9-10 a.m.	2	12.8	9.2
2- 3 p.m.	5	29.8	15.0
9-10 p.m.	5	33.4	16.6
2- 3 a.m.	2	17.7	12.6

period with a slightly lower value evident during the 2-3 p.m. interval. Minimum concentrations of corticosterone were found in the plasma obtained from animals at 2-3 a.m. and 9-10 a.m. There was little variation in levels of the F-like material in the blood of the female animals obtained at the 2-3 p.m. and 9-10 p.m. intervals.

The presence of a diurnal rhythm in activity of the adrenal gland of the mouse was shown by variation in levels of corticosterone in the blood. The highest concentration was found during the afternoon(3). Similar results were obtained in the study on the rat(6) where maximum concentrations of corticosterone were observed between 4 p.m. and midnight. In the data reported here, the highest levels of corticosterone, in both male and female rats, occurred between 9-10 p.m. with somewhat lower levels during the 2-3 p.m. interval. Though there is some difference in time of illumination in this study and that used by Guillemin *et al.*(6), these data support the evidence that there is a diurnal variation in concentration of corticosterone in the blood of the rat with a maximum activity during early afternoon and evening. Interestingly enough, no diurnal rhythm was found for the F-like material in the plasma of the rat. These results tend to indicate that the F-like component does not respond to the day-night stimulus as seen with corticosterone and supports the concept that corticosterone is the glucocorticoid of physiological significance in the rat.

Summary. Concentration of corticosterone in blood of the rat varied as a function of time of day. Highest levels of corticosterone were found between 2 and 3 p.m., and 9 and 10 p.m. There was no diurnal variation in concentration of an unknown component of adrenal origin found in blood. These data supply additional evidence for presence of a diurnal rhythm in secretory activity of the adrenal cortex and support the concept that corticosterone is the glucocorticoid of physiological significance in the rat.

Samples of corticosterone and 17-hydroxycorticosterone were supplied through courtesy of Merck, Rahway, N. J., and Upjohn Co., Kalamazoo, Michigan.

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Received July 7, 1960. P.S.E.B.M., 1960, v104.

7